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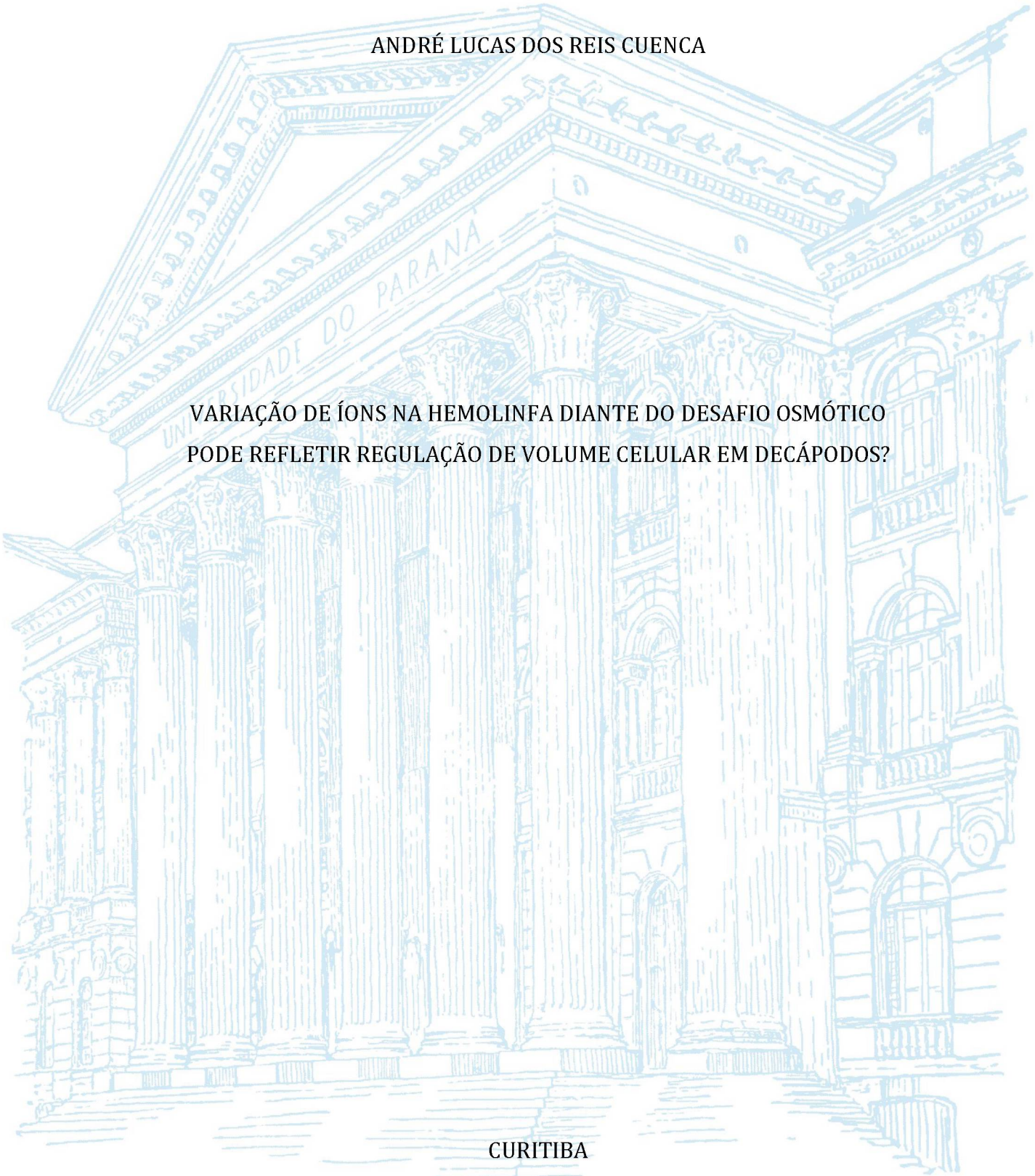

# VARIAÇÃO DE ÍONS NA HEMOLINFA DIANTE DO DESAFIO OSMÓTICO PODE REFLETIR REGULAÇÃO DE VOLUME CELULAR EM DECÁPODOS?

CURITIBA

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Dissertação apresentada ao Programa de Pós-Graduação em Zoologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Zoologia.

Orientadora: Prof.ª Dra. Carolina Arruda de Oliveira Freire

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# TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **ANDRÉ LUCAS DOS REIS CUENCA** intitulada: **Variação de íons na hemolinfa diante de desafio osmótico pode refletir regulação de volume celular em decápodos?**, sob orientação da Profa. Dra. CAROLINA ARRUDA DE OLIVEIRA FREIRE, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 20 de Fevereiro de 2020.

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Giovanna C. Castellano.
GIOVANNA CARSTENS CASTELLANO
Avaliador Externo (FACULDADES SANTA CRUZ )

MARTA MARQUES DE SOUZA
Avaliador Externo (UNIVERSIDADE FEDERAL DO RIO GRANDE)

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*Man, he took his time in the sun*

*Had a dream to understand*

*A single grain of sand*

Nightwish – The Greatest Show on Earth

## RESUMO

Crustáceos decápodos são um grupo zoológico bastante diversificado, que ocupa ambientes marinhos, estuarinos, dulcícolas e semi-terrestres. Tipicamente, decápodos marinhos são osmoconformadores, enquanto aqueles que conquistaram ambientes diluídos são osmorreguladores. Osmorreguladores promovem o transporte de íons entre a hemolinfa e o ambiente, tamponando as flutuações na osmolalidade causadas por qualquer variação na salinidade da água, através da regulação anisosmótica extracelular (AER). Ainda assim, em diferentes intensidades, desafios de salinidade também levam ao fluxo de íons entra a hemolinfa do animal e o compartimento intracelular, e a regulação chamada de isosmótica intracelular (RII) atua na regulação do volume celular durante esses desafios, dado que alterações de água e solutos no extracelular rapidamente se comunicam com o intracelular. Assim, o perfil iônico da hemolinfa de um decápodo submetido a um desafio salino pode ser influenciado tanto pela AER quanto pela RII, dependendo da estratégia adotada, mecanismos disponíveis, o quê por sua vez reflete o ambiente e história evolutiva do decápodo. Assim, estudamos quatro espécies de decápodos (o camarão marinho *Litopenaeus vannamei*, o siri marinho/estuarino *Callinectes danae*, o camarão diádromo *Macrobrachium acanthurus* e o anomuro hololimnético *Aegla schmitti*), com diferentes histórias evolutivas de invasão ao ambiente dulcícola, para avaliar em que grau a RII pode influenciar as concentrações iônicas da hemolinfa. As espécies marinhas foram submetidas a um desafio hipo-salino (de 25 e 30‰ em *L. vannamei* e *C. danae,* para 15‰), enquanto as espécies de água doce foram submetidas a um desafio hiper-salino (água doce para 25‰), ambos por 5 e 10 dias, para restringir a influência de mecanismos de escape e permitir tempo suficiente para que estados estacionários fossem alcançados. O hiper-hipo-regulador *L. vannamei* apresentou redução de apenas 12% da osmolalidade da hemolinfa, mas o teor hídrico muscular aumentou 5%, uma possível consequência da ausência de efluxo relevante nas concentrações iônicas no músculo. O hiper-regulador fraco/conformador *C. danae* teve uma redução de ~25% da osmolalidade da hemolinfa, mas o teor hídrico foi estável. As concentrações de sódio e cloreto no tecido foram reduzidas, mas as de potássio permaneceram inalteradas, o que pode indicar um efeito sinergístico dos canais de potássio e cloreto com a $Na^+/K^+$-ATPase para promover o efluxo de íons e efetuar a redução de volume celular em condição hipo-osmótica. *M acanthurus* hipo-regulou fortemente no desafio hiper-salino, mantendo teor hídrico inalterado, garantindo uma razão estável da concentração total de osmólitos no tecido para a osmolalidade da hemolinfa. O anomuro *A. schmitti* osmoconformou no desafio de longa duração, com uma aparente hipo-regulação de cloreto. Para manter a hidratação tecidual, essa espécie acumulou todos os solutos analisados, com um destaque para o cloreto. Assim, a aparente hipo-regulação de cloreto na hemolinfa ocorre pois *A. schmitti* pode promover um forte influxo do íon para os tecidos na manutenção da integridade celular. Nossos resultados sugerem que conclusões sobre o perfil osmorregulatório de decápodos - em geral considerando as alterações extracelulares como sendo apenas produto da AER - quando submetidos a fortes desafios de salinidade, devem ser feitas com cuidado, uma vez que a RII pode na verdade enviesar as concentrações hemolinfáticas, levando a conclusões imprecisas sobre a capacidade de AER nesses animais. Os resultados, de forma geral, nos permitem responder "sim" à pergunta feita no título deste trabalho.



**Palavras-chave:** Regulação Isosmótica Intracelular. Crustáceos. Osmorregulação. NKCC.

## ABSTRACT

Decapod crustaceans are a widespread zoological group, occupying marine, estuarine, freshwater and semi-terrestrial environments. Tipically, marine decapods are osmoconformers, while those who conquered diluted environments osmoregulators. Osmoregulators promote the transport of ions through their hemolymph and the surrounding environment, buffering fluctuations on its osmolality caused by any change water salinity, via the anisosmotic extracellular regulation (AER). Nonetheless, at different degrees, salinity challenges also lead to the flux of ions between the animal's hemolymph and the intracellular compartment, and the isosmotic intracellular regulation (IIR) mechanisms aid on the maintenance of cell volume regulation during these challenges, since extracellular alterations on water and solutes rapidly communicate with the intracellular compartments. In this sense, the ionic profile of a decapod submitted to a salinity challenge might be influence by both AER and IIR, depending on the strategy in place and available mechanisms, which reflects environmental adaptations and evolutionary history of the decapod. Here, we studied four decapod species (the marine shrimp *Litopenaeus vannamei*, the estuarine crab *Callinectes danae*, the freshwater diadromous prawn *Macrobrachium acanthurus* and the freshwater homolimnetic anomuran *Aegla schmitti*), with different evolutionary histories of invasion of freshwater environments, to evaluate at what extent IIR might be influencing the ionic concentrations of their hemolymphs. The marine species were submitted to hypo-saline challenges (from 25 and 30‰ for *L. vannamei* and *C. danae*, to 15‰), and the freshwater ones to hyper-saline challenges (from freshwater to 25‰), for 5 and 10 days. The hyper-hypo-regulator *L. vannamei* displayed hemolymph dilution of only 12%, but muscle hydration increased 5%, a possible consequence of the lack of solute efflux from the tissue. The weak hyper-regulator/conformer *C. danae* showed a reduction of ~25% of hemolymph osmolality, but tissue hydration was stable. Muscle levels of both sodium and chloride lowered, but potassium remained unchanged, which could indicate a possible synergy between chloride and potassium channels with the $Na^+/K^+$-ATPase to reduce cell volume and keep tissue hydration on hypo-osmotic conditions. *M. acanthurus* strongly hypo-regulated on the hyper-saline challenge, and showed stable muscle hydration as well, keeping steady ratios of total muscle concentrations to hemolymph osmolality. The anomuran *A. schmitti* osmoconformed to the water at the longest hyper-saline challenge, but an apparent hypo-regulation of chloride was observed. To keep steady muscle hydration, this species accumulated all of the assessed osmolytes on the tissue, and chloride increased the most. In this sense, the apparent hypo-regulation of hemolymph chloride is because *A. schmitti* could actually be promoting the ion's influx to tissue at a strong pace, to preserve cell volume and integrity. Our results suggest that osmoregulatory profiling of decapods submitted to challenging salinities should be approached with care, since strong IIR mechanisms might actually shift hemolymph ionic gradients, misleading conclusions on the AER capability of these animals. In general, results allow us to answer "yes" to the question posed on the title of this work.



**Keywords:** Isosmotic Intracellular Regulation. Crustaceans. Osmoregulation. NKCC.

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**SUMÁRIO**

## PRÓLOGO

Os crustáceos da ordem Decapoda ocupam vários ambientes aquáticos, sendo encontrados no mar, em estuários e em água doce. Nos ambientes marinhos, grande parte dos decápodos são osmoconformadores, ao passo que em ambientes diluídos, em especial em água doce, predominam os osmorreguladores (Péqueux, 1995). A manutenção de um gradiente osmótico entre o meio extracelular e o meio externo foi de fundamental importância na invasão do ambiente dulcícola, dada a escassez de sais nesse ambiente (Kirschner, 1991; Freire et al., 2008b).

Por meio da regulação anisosmótica extracelular, os organismos osmorreguladores são capazes de absorver ou excretar ativamente os sais do e para o meio (através dos epitélios osmorregulatórios, em especial as brânquias), mantendo a osmolalidade do líquido extracelular em uma faixa de tolerância adequada ao bom funcionamento dos tecidos e garantindo um menor desafio à manutenção do volume de suas células (Florkin, 1962; McNamara & Faria, 2012). Por outro lado, osmoconformadores apresentam osmolalidade da hemolinfa semelhante à do ambiente e, portanto, dependem da ativação de mecanismos de regulação isosmótica intracelular para manter estável o volume de suas células em situações de desafios de salinidade (Kirschner, 1991, 2004; Foster et al., 2010).

Variados perfis osmóticos podem ser encontrados em Decapoda, em que os animais podem responder diferentemente de acordo com a salinidade e/ou tempo de exposição à determinada situação (Foster et al., 2010). Como em muitos casos não há uma clara distinção entre comportamentos estritamente osmorregulatórios ou osmoconformadores (Freire et al., 2003, 2008a), estima-se que ambos os mecanismos de regulação anisosmótica extracelular e isosmótica intracelular ajam concomitantemente. Desta forma, é de grande importância considerar não apenas as alterações em concentrações da hemolinfa que um animal exibe diante do desafio osmótico, mas também de seu meio intracelular, visto que parte da variação observada pode ser referente à manutenção do volume celular, imprescindível para seu bom funcionamento (Bozza et al., 2019).

Assim, o presente trabalho buscou identificar – considerando quatro linhagens com histórias evolutivas de conquista da água doce e distribuições distintas – como as concentrações extra- e intracelulares se relacionam nesses decápodos que enfrentam desafios osmóticos.

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## CAPÍTULO ÚNICO

(Formatação: *Comparative Biochemistry and Physiology, Part A*)

# Does the variation in hemolymph ions upon osmotic challenge reflect cell volume regulation in decapods?

CUENCA, André L. R.[1]; SOUZA, Marta M.[2]; FREIRE, Carolina A.[3]

[1]Programa de Pós-graduação em Zoologia, Universidade Federal do Paraná

[2]Instituto de Ciências Biológicas, Universidade Federal do Rio Grande

[3]Departamento de Fisiologia, Setor de Ciências Biológicas, Universidade Federal do Paraná

## 1. INTRODUCTION

Decapod crustaceans are widespread throughout marine, estuarine, freshwater and terrestrial environments, and their conquest of diluted waters is tightly related to osmoregulation, specifically to anisosmotic extracellular regulation (AER) (Péqueux, 1995; Freire et al., 2008b; Foster et al., 2010). Species who perform AER are capable of actively absorbing or secreting salt through osmoregulatory epithelia such as gills (most importantly) and antennal glands (Florkin, 1962; Freire et al., 2008b; McNamara and Faria, 2012), thus maintaining extracellular fluid (ECF) osmolality at fairly stable ranges when facing osmotic challenges. On the other hand, marine species with a long evolutionary history on such stable environments - in fact, ancestral state for decapods - are mostly osmoconformers, unable to perform AER. Therefore, salinity changes on the environment are reflected on ECF – or hemoymph – osmolality (Péqueux, 1995; Foster et al., 2010). Such disturbances pose a challenge on tissue cells' volume, in which active isosmotic intracellular regulation (IIR) mechanisms promote osmolyte (and hence water) flow between extra- and intracellular media (Amado et al., 2006; Faria et al., 2011), counteracting osmotic disturbance and keeping cell volume at steady levels.

Species that are said to osmoregulate actually display AER to various degrees. Their euryhalinity, or tolerance to salinity challenges, is tightly related to mode (abrupt/gradual) and time of exposure, and the intensity of the osmotic gradient posed (Kirschner, 1991; Péqueux, 1995; Freire et al., 2003, 2008a). In this sense, responses to

osmotic challenges can be variable, and mostly do not follow the restrictive and limits of the mutually exclusive "osmoconformer *vs.* osmoregulator" definitions.

Euryhalinity on decapod osmoconformers, however, despite being in general much narrower than in osmoregulators, is strongly associated to a greater capacity of IIR (Foster et al., 2010). Upon exposure to salinity decrease – as conformers are typically very permeable to water and ions - ECF osmolality is in consequence reduced, and tissue cells tend to swell due to water influx. Normal volume is recovered by regulatory volume decrease (RVD), which consists of efflux of osmolytes from the cells to the ECF, accompanied by water. In the opposite direction, increased ECF osmolality as a result of exposure to higher salinity leads to cell shrinkage due to water loss. Regulatory volume increase (RVI) mechanisms of osmolytes influx are then activated, to which, again, water flow follows and restores cell volume (Amado et al., 2006, 2015; Santos et al., 2013; Castellano et al., 2016). These mechanisms function both initially on the transport of inorganic ions, followed by the metabolism of organic osmolytes, especially free aminoacids, given that disturbances on intracellular inorganic concentrations may interfere with metabolic pathways (Charmantier et al.; 2009). Considering that the mechanisms of IIR are well-conserved across evolutionary history, its activation upon osmotic challenges is considered a plesiomorphic response (Freire et al., 2008a; Faria et al., 2011) when compared to AER.

As a variety of responses to osmotic challenges are observed across lineages of decapod crustaceans, both AER and IIR mechanisms are expected to work together in the maintenance of cell volume at different degrees, in relation to the species habitat distribution and thus its evolutionary history (Faria et al., 2011; McNamara and Faria, 2012). In this sense, when evaluating how a species responds to osmotic challenges solely analysing its ECF osmolality and ionic profile may miss an important component, given that ECF is actually the intermediate compartment between the cells and tissues and the external medium. Thus, additional care should be taken, since IIR mechanisms – maintenance of cell volume at steady levels – consists in the transport of osmolytes between extra- and intracellular media, thus potentially interfering with ECF ionic concentrations and osmolality (Figure 1). Therefore, changes that would be interpreted as exclusively hypo- or hyper-regulation (AER, fluxes between ECF and the external

medium) could be, at least partially, related to IIR, or fluxes between ECF and ICF (Foster et al., 2010; Freire et al., 2013; Bozza et al., 2019).

Here, we aim at identifying how the changes in ECF ionic concentrations during osmotic challenges could be related not only to AER, but also to IIR. We analysed both ECF and intracellular fluid concentrations and have evaluated four decapod species from different lineages and habitats: the penaeid shrimp *Litopenaeus vannamei*, the marine/estuarine portunid crab *Callinectes danae*, the diadromous palaemonid prawn *Macrobrachium acanthurus*, and the hololimnetic anomuran aeglid *Aegla schmitti*.

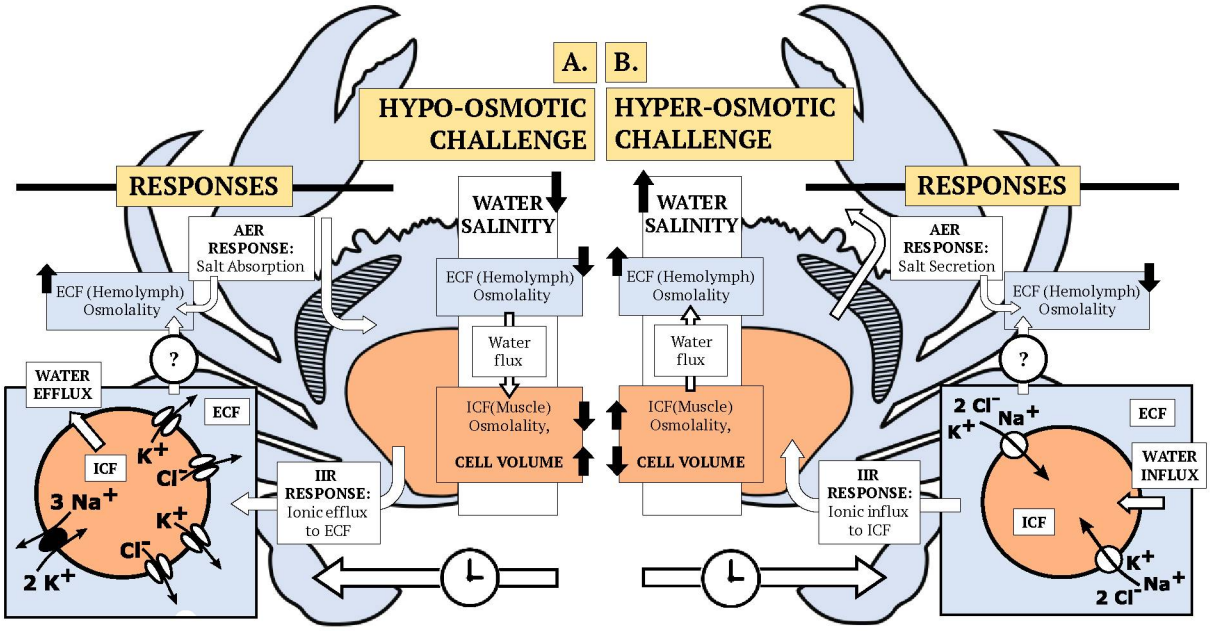


**Figure 1.** Summary of the effects of and responses to osmotic challenges on a generic, osmoregulating decapod crustacean. Hypo- (**A**, left) and hyper-osmotic (**B**, right) challenges have opposite effects on the extracellular fluids (ECF or hemolymph, light blue), which affects, in different degrees, the intracellular fluid (ICF, orange). In response to hypo-osmotic challenges, the Anisosmotic Extracellular Regulation (AER) increases salt absorption through gills (stripes), while tissues (in this case, muscle) performing Isosmotic Intracellular Regulation (IIR) secreting ions (through chloride and potassium channels, reversible NKCC cotransporter and $Na^+/K^+$-ATPase). Conversely, the AER response to hyper-osmotic challenge is mainly salt secretion through gills, and the IIR response of tissues is ion absorption, through NKCC. Interrogation symbols represent the question posed on the title of this work: is IIR influencing ECF ionic concentrations?

# 2. MATERIAL AND METHODS

## 2.1. Animals

The choice of animals in this study aimed at reflecting the evolutionary trend of freshwater invasion observed in crustaceans (Péqueux, 1995; Freire et al., 2003; Foster et al., 2010; McNamara and Faria, 2012), especially in Decapoda, encompassing marine to hololimnetic representatives, albeit from distinct lineages and extant habitats.

*Litopenaeus vannamei* (Dendrobranchiata, Penaeidae) is a hyper/hypo-regulator shrimp, with coastal and marine occurrences. In fact, different representatives of Penaeidae are considered able to sustain very large osmotic gradients, as pointed out by Castille and Lawrence (1981). This species has a major economical relevance, being the most cultivated shrimp worldwide (Zhou et al., 2011). *L. vannamei* is very euryhaline, and has an isosmotic point close to 25‰ (~750 mOsm/kg $H_2O$), and significantly hyper-regulates in diluted environments (although not in freshwater), and hypo-regulates in seawater, thus always maintaining its ECF osmolality at narrow ranges, despite environmental variation (Gomez-Jimenez et al., 2004; Huong et al., 2010; Zhou et al., 2011; Liu et al., 2016).

*Callinectes danae* (Brachyura, Portunidae) is a common species of swimming crab in brackish waters of southern and south-eastern bays of Brazil, abundant on the inner part of estuaries (with lower salinities) but occurring from seawater to oligohaline water of 9‰ salinity (Severino-Rodrigues et al., 2009). Ovigerous females usually migrate to the open sea for spawning (Mantelatto and Fransozo, 2000). *C. danae* is an euryhaline species, although also incapable of inhabiting freshwater environments, being thus a typical "weak" hyper-regulator, actively taking up sodium and chloride from brackish water (Freire et al., 2008b; Charmantier et al., 2009; Henry et al., 2012). Also, in full-strength seawater, *C. danae*, as other weak regulators, osmoconforms, being essentially isosmotic to marine osmolality, or else sustaining small gradients (Masui et al., 2005, 2009; Freire et al., 2011). Other species of *Callinectes* have different patterns of distribution on the estuary, where *C. ornatus* is found on polyhaline waters (Freire et al., 2011), while *C. sapidus* can reach the most diluted portions of the estuary and even freshwater (Ettinger and Blye Jr, 1981).

*Macrobrachium* (Caridea, Palaemonidae) is a diverse and widespread genus on freshwater environment with more than 240 species. Lineages of Palaemonidae are considered recent freshwater invaders, and its diversification – particularly *Macrobrachium* – dates from the late Oligocene to early Miocene (McNamara and Faria, 2011; Faria et al., 2011; Collins et al., 2011; Freire et al., 2013, 2018). Given its recent history of freshwater invasion, the genus presents hololimnetic species like *M. potiuna* (Freire et al., 2003), but also diadramous ones, like *M. acanthurus* and *M. olfersi*, which depend on brackish water for larval and early-stage development (Albertoni et al., 2002; Foster et al., 2010). The high degree of euryhalinity of *M. acanthurus* has already been recorded multiple times, and the species can survive for at least 5 days on waters of 20‰ salinity (Freire et al., 2018), at least 24 h in 25‰ (Maraschi et al., 2015), or up to 12 h in full-strength seawater (Foster et al., 2010).

The fourth species used, the anomuran *Aegla schmitti* (Aeglidae), belongs to an ancient freshwater coloniser genus, endemic to South America, which originated from the Pacific during the late Cretaceous. Its freshwater radiations first occurred around 40 million years ago (Collins et al., 2011). Despite being the only extant hololimnetic anomuran lineage (Pérez-Losada et al., 2004), still little work has been done evaluating how aeglids respond to increased salinity, or how they deal with 'ancestral' salinities. Available data show that *A. franca* survives at 28‰ salinity for at least 5 days (Faria et al., 2011), and *A. schmitti*, the species used here, survives in 25‰ salinity also for at least 10 days, with moderate changes on hemolymph osmolality, seemingly hypo-regulating sodium and chloride at all times: 478-806 mOsm/kg $H_2O$ (Bozza et al., 2019).

## 2.2. Collection, Maintenance and Acclimation

*L. vannamei* shrimp (total length = 9.2 ± 0.7 cm; 9.5 ± 1.8 g) were acquired from the Marine Aquaculture Station (32°12'14.1"S; 52°10'40"W) of Federal University of Rio Grande, Rio Grande municipality, Rio Grande do Sul State. *C. danae* (carapace width = 10 ± 1 cm; 61 ± 16 g) were caught using baited net traps in an estuary at Pontal do Sul municipality (25°34'0.7"S; 48°21'18"W), Paraná State. *M. acanthurus* (total length = 4.9 ± 0.6cm, 2 ± 1 g) were bought from fishermen also in Pontal do Sul, where they are commonly sold as live bait after being collected from local streams (25°36'26"S;

48°24'3"W). *A. schmitti* (carapace length = 1.72 ± 0.27 cm; 1.82 ± 0.75 g) were collected using pond nets and kick/sweep sampling method in Rio Capivari II (25°9'57.22"S; 49°6'46.5"W), at Bocaíuva do Sul municipality, Paraná State. *L. vannamei* were transported to the laboratory at the Federal University of Rio Grande, in Rio Grande, while *C. danae*, *M. acanthurus* and *A. schmitti* were transported to the laboratory at the Federal University of Paraná in Curitiba, Paraná, where experiments and samplings took place.

Animals were acclimated for a minimum of 5 days in aquaria with water of salinity 25‰ (Refractometer RTS-101ATC, Instrutherm, Brazil) for *L. vannamei* (~100 L), 30‰ for *C. danae* (~100 L), and 0‰ salinity (30 L plastic boxes) for *M. acanthurus* and *A. schmitti*. All under constant filtering and aerating water, natural photoperiod and controlled room temperature (20 ± 2° C). Animals were fed with fish fillet or beef liver every other day and feeding was ceased 24 h prior to all experiments.

### 2.3. Experimental Design and Sampling

After acclimation, *L. vannamei* and *C. danae* were individually submitted to a hypo-osmotic shock in 15‰ salinity, for 5 and 10 days, in 3 L aquaria with 2.5 L of water. Conversely, freshwater species *A. schmitti* and *M. acanthurus* were submitted to a hyper-osmotic shock in salinity 25‰, also for 5 and 10 days, in 2 L aquaria containing 1.5 L of water. Natural, full-strength seawater was diluted with dechlorinated, filtered (cellulose and activated charcoal filters) tap water. Aquaria were provided with constant aeration throughout the experiments, and in all conditions, system water was renewed every other day in order to avoid ammonia build-up. Animals were not fed during the experimental period. An adequate number of independent replicates were conducted (n=7-10) for each species. Animals that were kept in stock acclimation aquarium were directly sampled as control groups, for each species, also considering the same fasting period as for the experimental groups.

Osmotic challenges and exposure times were chosen based on previous studies (Faria et al., 2011; Maraschi et al., 2015; Bozza et al., 2019), in order to provide consistent and comparable data to test our hypothesis. The relatively long periods of exposure chosen for this study (and based on the works aforementioned) aimed at representing a steady state condition under the respective osmotic challenge, avoiding the influence of any particular behavioural or early-stage response from the animal.

After the experimental period had elapsed, animals were anesthetised in ice (5 to 10 min, depending on animal size), measured (total length for shrimps, carapace length for *A. schmitti*, and carapace width for *C. danae*) and weighed. Hemolymph was sampled after a quick and total removal of the carapace (for *C. danae*), or by puncturing the arthrodial membrane with a micropipette (for both species of shrimps), or using an insulin syringe (for *A. schmitti*). Muscle samples were obtained from the abdominal muscle (shrimps and *A. schmitti*) or from the base of pereiopods (*C. danae*). All samples were stored at -20° C until further analyses.

### 2.4. Hemolymph Determinations

Hemolymph osmolality was read using a vapour pressure micro-osmometer (Vapro 5520, Wescor, USA). Sodium and potassium concentrations were determined by flame photometry (Digimed DM-62, Brazil); chloride and magnesium were analysed colorimetrically using commercial kits (LabTest, Brazil), with absorbance read at 450 and 505 nm, respectively. Hemolymph and muscle homogenates were diluted as needed, so that absorbances would remain within the limits of linearity of the assays.

### 2.5. Muscle Water and levels of Inorganic and Organic Osmolytes

Muscle samples were weighed using an analytical scale (0.1 mg precision, Bioprecisa FA2104 N, Brazil), dried at 60-64° C for 48 h and weighed again, thus obtaining water content as a percentage of sample's wet weight. After, inorganic ion content extraction was done as described by Amado et al. (2006), which briefly consists of digestion of previously dehydrated muscle samples in nitric acid (0.75 N) for 48 h at room temperature. After centrifugation (5000×g for 5min), supernatant was stored and ion levels were analysed as previously described for hemolymph samples. Muscle organic osmolytes were determined using the protocol for ninhydrin-positive substances (NPS), adapted from Clark (1968) for small samples, and as described in Foster et al. (2010) and Castellano et al. (2017).

It is important to mention that the protocols here used to determine muscle inorganic osmolytes concentrations cannot prevent contamination from the intrinsic extracellular space of the tissue, thus leading to the introduction of a systematic error in the data generated. As an outcome, sodium and chloride are likely to be overestimated, while potassium underestimated. Therefore, absolute values provided in this work may

not reflect accurate intracellular concentrations, but the direction of the changes observed are assured, which was essentially the goal of the study.

### 2.6. Data Analysis

Direct descriptions of both hemolymph and muscle inorganic ion concentrations were expressed as mmol/L (mM), to ensure direct comparisons between them. To do so, the latter was calculated as millimoles per liter of tissue (muscle) water content (mmol/L of MWC) as described by the formula below.

$$[\text{ion}]_{\text{muscle}} \left(\frac{mmol}{L\ of\ MWC}\right) = \frac{[\text{ion}]_{\text{homogenate}}(mmol/L) \times \text{volume of } HNO_3(L)}{(\text{sample fresh weight } (kg) \times \text{MWC } (\%)) \div 100}$$

NPS concentrations were expressed as µg/mg of fresh weight, based on a standard curve of glycine (2.5 – 50 mg/mL) designed with the same protocol.

Contribution to intracellular – tissue – osmolality (CTO, %) was calculated based on McNamara et al. (2004) and Faria et al. (2011), considering osmotic equilibrium between muscle tissue (ICF) and hemolymph (ECF), which is expected to happen quickly in animal cells. Concentrations were converted from mmol/L to mmol/kg fresh weight, and the contribution was calculated as follows below. To convert NPS concentration from weight to moles on this specific analysis, we considered the average molecular weight of the main amino acids present on decapod crustaceans (glycine, taurine, alanine and arginine, average of~115 g/mol, Cobb et al., 1975; Wheatly, 1985; Faria et al., 2011; Zhou et al., 2011).

$$\text{CTO } (\%) = \frac{[\text{ion}]_{\text{muscle tissue}}\ (mmol/kg\ fresh\ weight)}{\text{hemolymph osmolality } (mOsm/kg\ H_2O)}$$

Values were not corrected considering the interference of extracellular space of tissue sampled. Contribution of magnesium to tissue osmolality never surpassed 3%, so it was considered negligible on this particular analysis. This appropriately named index aim to identify the contribution of each intracellular component to the iso-osmotic balance between ICF and ECF.

Total concentration of ions in muscle was calculated as the sum of sodium. chloride, potassium and magnesium (also expressed in mM), and was also used to calculate its ratio to hemolymph osmolality.

As a proxy to the interference of IIR ionic fluxes on detected hemolymph ionic concentrations, an index was calculated as described by the formula below, simply called Regulatory Index (RI) for further references herein. The index is expressed as a ratio between the relative variation of the ion on the muscle, to the relative variation of the ion on the hemolymph, considering the osmotic challenges proposed. Values close to one indicate that both muscle and hemolymph concentrations varied in accordance; while values higher or lower than one indicate a greater variation on muscle or hemolymph levels, respectively.

$$\mathrm{RI} = \frac{\Delta\%\,[\mathrm{ion}]_{\mathrm{muscle}}}{\Delta\%\,[\mathrm{ion}]_{\mathrm{hemolymph}}} = \frac{\left|\left([\mathrm{ion}]_{\mathrm{muscle}}^{\mathrm{challenge}} - [\mathrm{ion}]_{\mathrm{muscle}}^{\mathrm{control}}\right) \div [\mathrm{ion}]_{\mathrm{muscle}}^{\mathrm{control}}\right|}{\left|\left([\mathrm{ion}]_{\mathrm{hemolymph}}^{\mathrm{challenge}} - [\mathrm{ion}]_{\mathrm{hemolymph}}^{\mathrm{control}}\right) \div [\mathrm{ion}]_{\mathrm{hemolymph}}^{\mathrm{control}}\right|}$$

Statistical comparisons were made within each species, considering control and experimental treatments, using one-way ANOVA and Tukey as the *post-hoc* analysis. When necessary, Kruskal-Wallis ANOVA for non-parametric data with Dunn's *post-hoc* analysis was performed, always considering p-value limited at 0.05. These statistical analysis were made using Sigma Plot® v11 Software. Regression analyses were performed on R, for each species, in order to identify correlations between the concentrations of a specific ion in the hemolymph and in muscle tissue.

## 3. RESULTS

Hemolymph osmolality and muscle water content of the four decapod species are summarized on the graph below (Figure 2) and described on their respective sections.

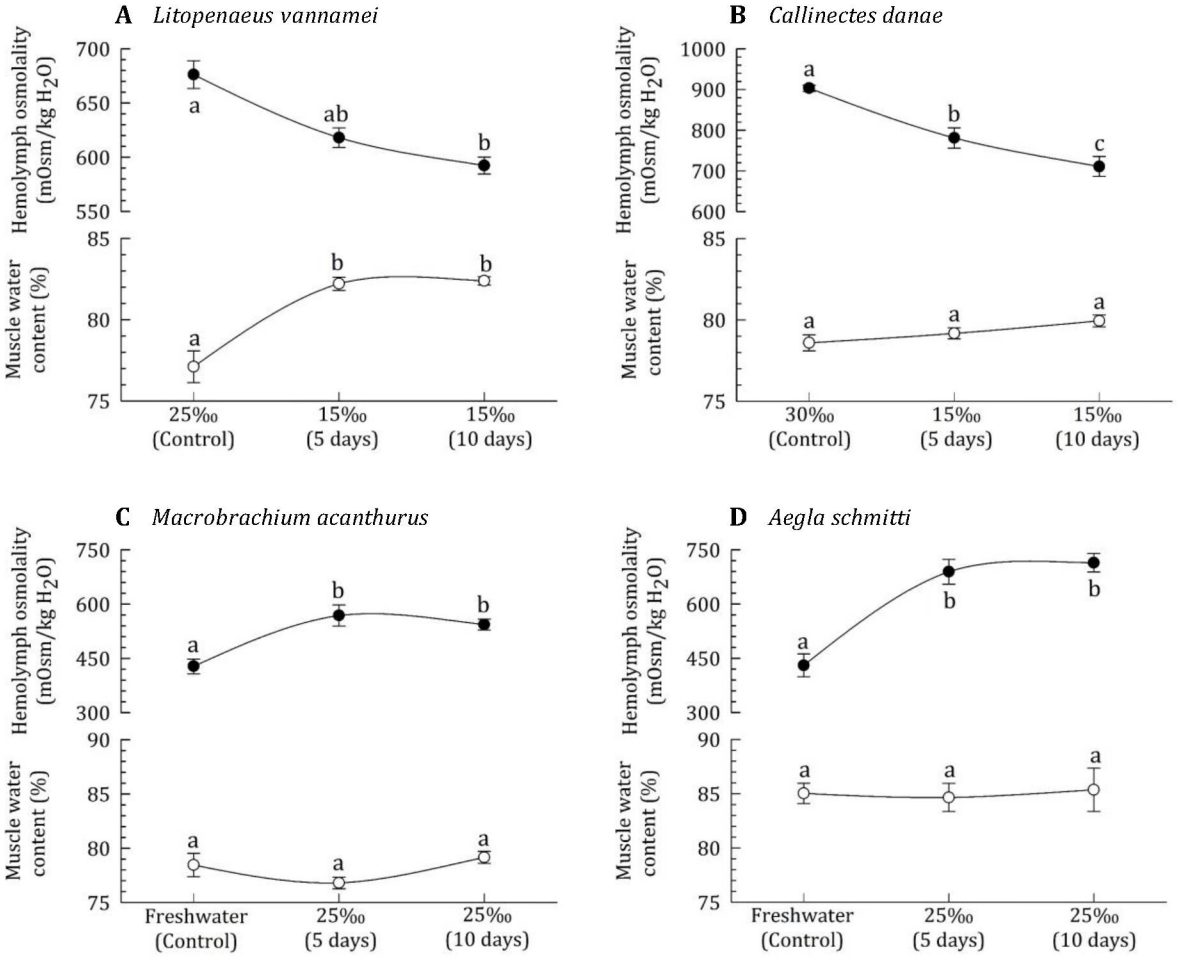


**Figure 2.** Hemolymph osmolality (mOsm/kg $H_2O$) and muscle water content (%) of the four crustacean species submitted to osmotic challenges. **A**: *Litopenaeus vannamei* (10≤n≤7); **B**: *Callinectes danae* (n=10); **C**: *Macrobrachium acanthurus* (n=10); **D**: *Aegla schmitti* (n=10). Different lowercase letters represent groups that are statistically different.

Mortality of individuals were 11.7% (4 out of 34) for *A. schmitti* and *M. acanthurus* and 16.6% (5 out of 30) for *L. vannamei*. No individuals of *C. danae* died during the experiments. Deaths were mostly associated with individuals that underwent moulting while exposed to the osmotic challenge.

### 3.1. *Litopenaeus vannamei*

Hemolymph osmolality of control shrimps was 676 mOsm/kg $H_2O$, reducing progressively to 618 and 592 mOsm/kg $H_2O$ after 5 and 10 days on 15‰ salinity, respectively. Muscle water content (MWC) increased due to the hypo-osmotic shock:

control shrimps presented muscle hydration of 77%, in contrast to 82% on both experimental groups (Figure 2A).

Hemolymph sodium ($[Na^+]_h$), also lowered, ~50 mM after 5 and 10 days on 15‰ (Figure 3A), while muscle sodium ($[Na^+]_m$) remained stable, of ~64 mM, but $\Delta[Na^+]$ varied from -238 mM (control) to -198 and -153 mM on each hypo-osmotic condition (Figure 4A). The ion's contribution to tissue osmolality (CTO) increased 3.7% (Table 1). Differently, chloride ($[Cl^-]_h$) concentrations remained stable, around 360 mM, while muscle chloride ($[Cl^-]_m$) increased ~11mM at experimental conditions (Figure 3B), maintaining $\Delta[Cl^-]$ = -275mM (Figure 4B), and contributing to ~10.9% of tissue osmolality at all groups (Table 1).

Slightly lower concentrations of hemolymph potassium ($[K^+]_h$) were detected on both experimental treatments, and muscle potassium ($[K^+]_m$) went from 108 mM in control shrimps to 85 and 98 mM after 5 and 10 days of exposure (Figure 5A) $\Delta[K^+]$ ranged from 98 to 78 mM (Figure 6A), but always contributing to ~12.4% of tissue osmolality.

Hemolymph magnesium ($[Mg^{2+}]_h$) levels were stable, ~5 mM, while muscle magnesium ($[Mg^{2+}]_m$) decreased to less than half its control value on experimental groups, being iso-ionic to $[Mg^{2+}]_h$ (Figures 5B and 6B). Last, muscle NPS were also stable (Figure 5B), remaining at ~23 µg/mg fresh weight (CTO ~ 33% at all groups, see Table 1).

Relative variations of inorganic ions further support the nominal changes described for each concentration. The RI suggest that for *L. vannamei*, variations on ICF ions were greater than on the ECF for sodium, chloride and magnesium (RI > 1, see Table 2). Total inorganic tissue concentration ($[Total]_m$) did not alter significantly throughout experimental conditions, with an average of 253 mM of analysed osmolytes and a ratio to hemolymph osmolality of 0.4 (see Table 1). The only significant correlation between hemolymph and muscle ions for *L. vannamei* was for sodium ($R^2$=0.2, $[Na^+]_m = -0.288 \times [Na^+]_h + 124.4$, see Figure 11A).

### 3.2. *Callinectes danae*

Hemolymph osmolality in control *C. danae* was of 903 mOsm/kg $H_2O$, diluting down 120-200 mOsm/kg $H_2O$ on diluted seawater. At all conditions, in spite of the reduction in hemolymph osmolality, MWC of *C. danae* remained at ~79%, (Figure 2B).

$[Na^+]_h$ also decreased, from 418 (control) to 358 and 321 mM after 5 and 10 days on diluted seawater. $[Na^+]_m$ lowered about 15 and 30 mM on the hypo-osmotic experiments (Figure 3C), but $[Na^+]_m$ always contributed to ~6.8% of muscle osmolality (Table 1). $\Delta[Na^+]$ decreased ~60 mM at experimental groups (Figure 4C).

A steep reduction of ~160 mM on $[Cl^-]_h$ could be observed only at the longest exposure time on diluted seawater, $[Cl^-]_m$ also reduced accordingly (only at the longest period), around ~38 mM (Figure 3D), which was reflected on reduced $\Delta[Cl^-]$ (Figure 4D) and CTO (see Table 1).

$[K^+]_h$ and $[K^+]_m$ were kept stable throughout all groups (10 and ~116 mM, respectively, Figure5C), and $\Delta[K^+]$ was of ~108 mM (Figure 6C). Potassium CTO lightly increased ~3% at the longest period on diluted seawater.

$[Mg^{2+}]_h$ lowered after 10 days only (14, 11 and 5 mM). $[Mg^{2+}]_m$ and NPS values were also steady throughout experiments, kept around 20 mM and 18 µg/mg, respectively (Figure 5D). In the control group, $[Mg^{2+}]_m$ was isoionic to $[Mg^{2+}]_h$, while a difference of ~8 mM was kept on both experimental groups (Figure 6D). NPS always contributed to ~20% of tissue osmolality.

The RI for *C. danae* support that $\Delta[Na^+]$% was slightly higher on muscle than on the hemolymph (RI = 1.2 and 1.5), and $\Delta[Cl^-]$% and $\Delta[K^+]$% presented the highest values, of 2.5 and 5.3, respectively, also showing that variations on ICF were much greater than on the hemolymph (Table 2).

$[Total]_m$ lowered ~240 mM in diluted seawater, while maintaining a ratio to hemolymph osmolality of 0.39 (see Table 1). A significant, positive correlation of sodium ($R^2$=0.58, $[Na^+]_m = 0.295 \times [Na^+]_h - 38.96$, Figure 11A) and chloride levels ($R^2$=0.26, $[Cl^-]_m = 0.149 \times [Cl^-]_h + 36.95$, figure 11B) on tissue and hemolymph could be drawn.

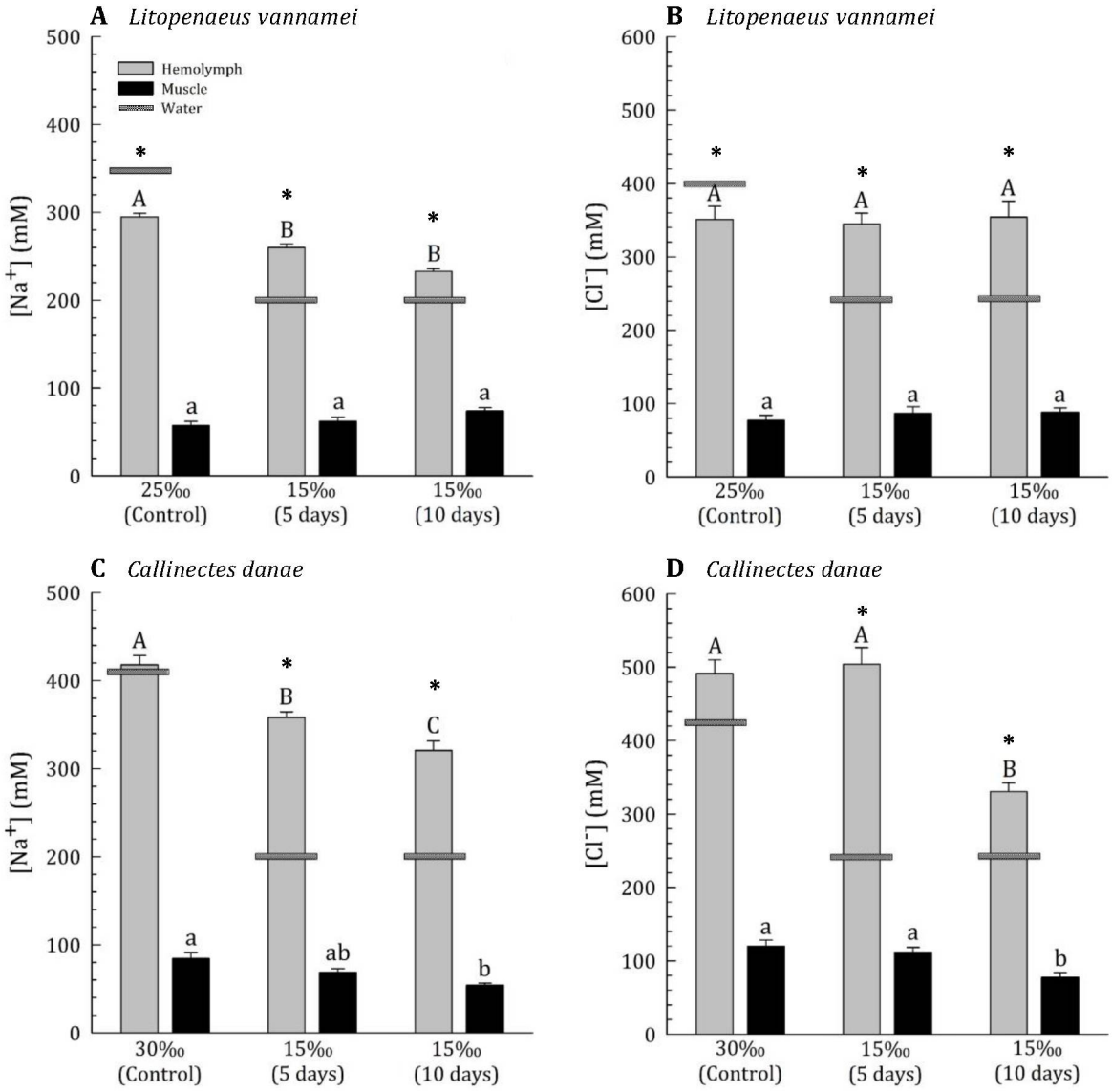


**Figure 3.** Profiling of sodium and chloride on hemolymph and muscle of *Litopenaeus vannamei* and *Callinectes danae* submitted to hypo-osmotic challenges. Sodium (**A, C**) and chloride (**B, D**) of *Litopenaeus vannamei* (10≤n≤7) and *Callinectes danae* (n=10), respectively. Different upper and lowercase letters stand for statistically different values for hemolymph and muscle concentrations. Horizontal marks are a reference to calculated concentration of the ion on the water, based on Prosser (1973), and asterisks indicate a significant difference between hemolymph and water concentrations, reflecting hyper- or hypo-regulation.

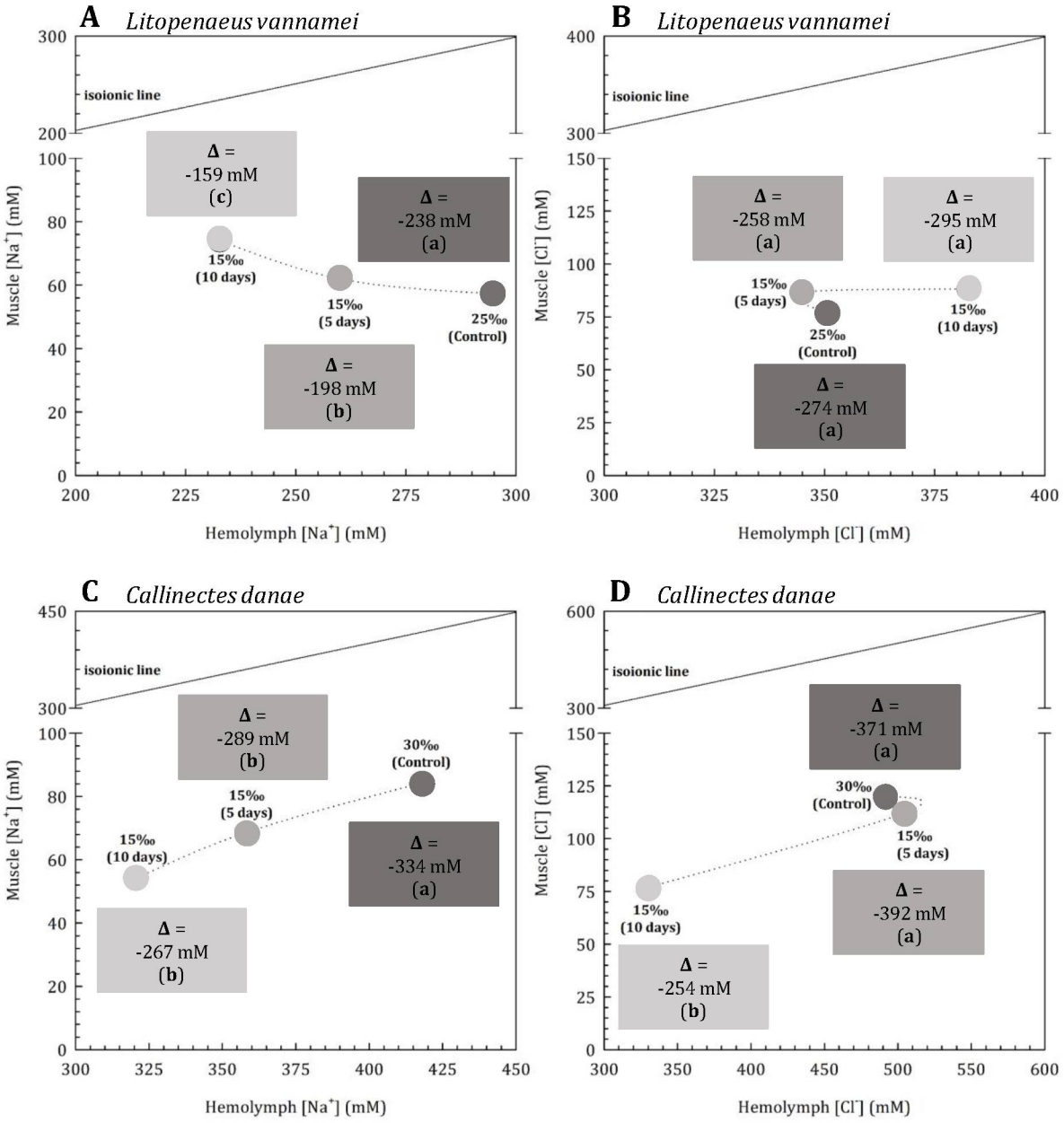


**Figure 4.** Differences of sodium and chloride between muscle and hemolymph of *Litopenaeus vannamei* and *Callinectes danae* submitted to hypo-osmotic challenges. Sodium (**A, C**) and chloride (**B, D**) of *Litopenaeus vannamei* (10≤n≤7) and *Callinectes danae* (n=10), respectively. Δ values are $[ion]_{muscle}$ – $[ion]_{hemolymph}$. Different lowercase letters stand for statistically different values of Δ among groups. Only mean values are expressed.

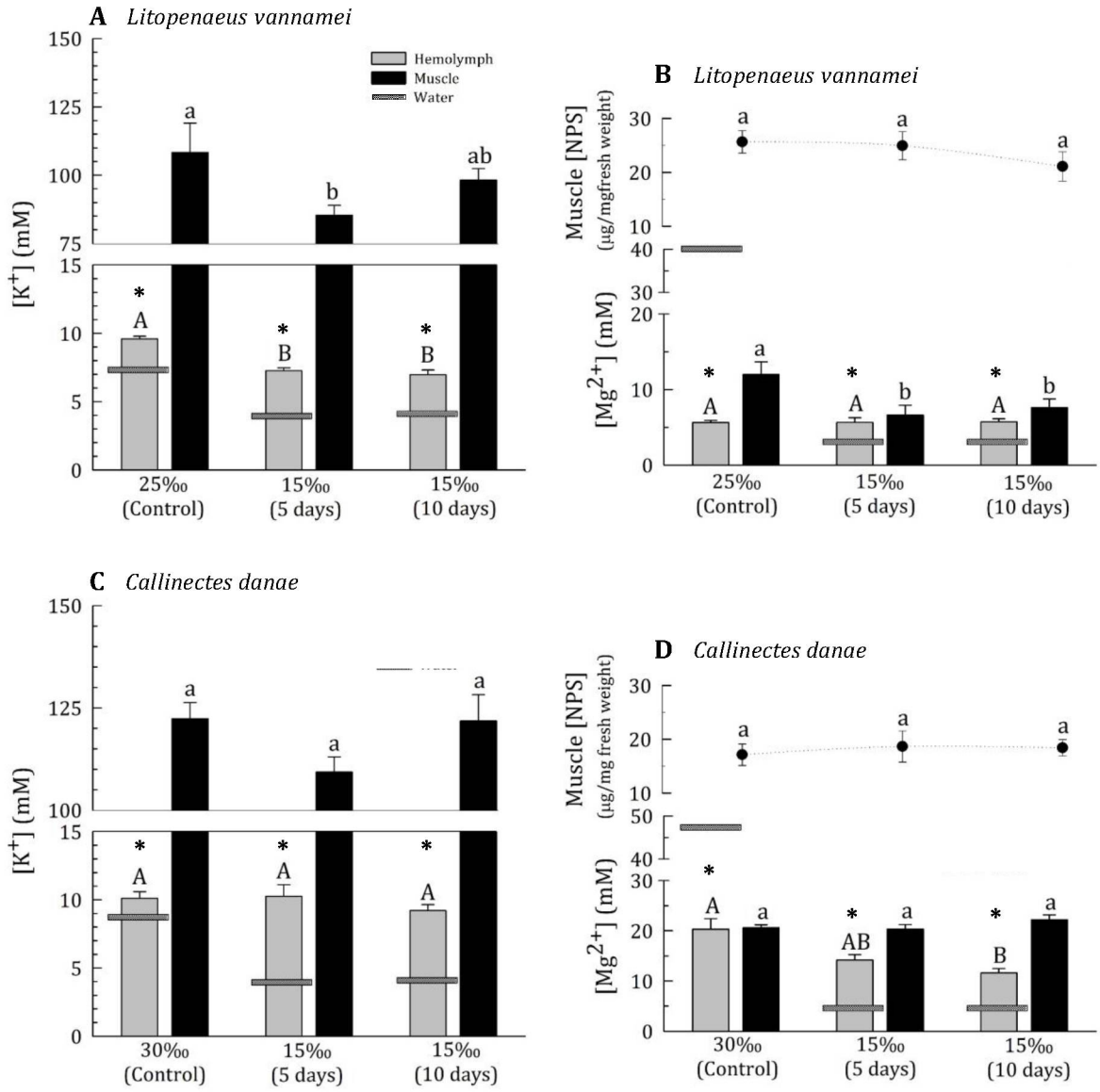


**Figure 5.** Profiling of potassium and magnesium on hemolymph and muscle (and muscle NPS) of *Litopenaeus vannamei* and *Callinectes danae* submitted to hypo-osmotic challenges. Potassium (**A, C**) and magnesium and NPS (**B, D**) of *Litopenaeus vannamei* (10≤n≤7) and *Callinectes danae* (n=10), respectively. Different upper and lowercase letters stand for statistically different values for hemolymph and muscle concentrations. Horizontal marks are a reference to calculated concentration of the ion on the water, based on Prosser (1973), and asterisks indicate a significant difference between hemolymph and water concentrations, reflecting hyper- or hypo-regulation.

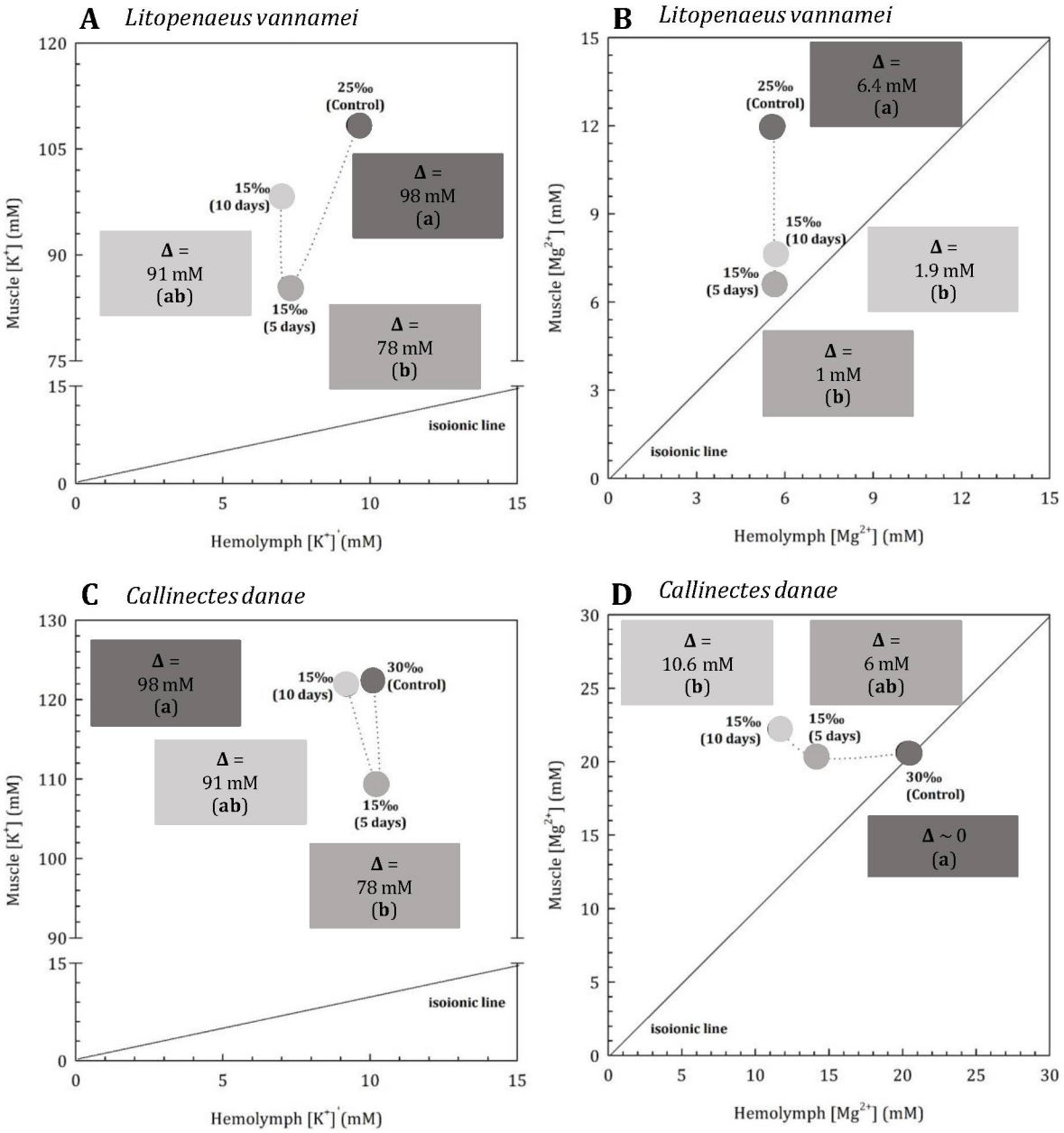


**Figure 6.** Differences of potassium and magnesium between muscle and hemolymph of *Litopenaeus vannamei* and *Callinectes danae* submitted to hypo-osmotic challenges. Potassium (**A, C**) and magnesium (**B, D**) of *Litopenaeus vannamei* ($10 \leq n \leq 7$) and *Callinectes danae* (n=10), respectively. Δ values are $[ion]_{muscle} - [ion]_{hemolymph}$. Different lowercase letters stand for statistically different values of Δ among groups. Only mean values are expressed.

### 3.3. *Macrobrachium acanthurus*

Hemolymph osmolality ranged from 430 mOsm/kg $H_2O$ (freshwater) to ~564 mOsm/kg $H_2O$ on shrimps exposed to brackish water. Tissue hydration of *M. acanthurus*, in all conditions, was kept around 78% (Figure 2C).

At all groups, no difference on $[Na^+]_h$ was observed, with an average of 250 mM. However, $[Cl^-]_h$ increased ~100 mM at both experimental conditions.$[Na^+]_m$ and $[Cl^-]_m$ also did not alter among treatments, being stable around 44 and 52 mM, respectively (Figures 7A and B). $\Delta[Cl^-]$ ranged from -121 mM (control) to -205 mM, while no difference was observed for $\Delta[Na^+]$ (Figure 8B). Sodium and chloride contributed to 6.8 and 7.9% of tissue osmolality, respectively.

$[K^+]_h$ and $[K^+]_m$ were stable around 9.3 and 130 mM, respectively, at all treatments (Figure 9A), which was reflected on steady $\Delta[K^+]$ (Figure 10A). Lower CTO at both exposures to 25‰ salinity (from 25.3% to ~17.7%).of $[K^+]_m$ was detected. $[Mg^{2+}]_h$ increased from 4.5 mM to 14 mM at both experimental groups, and $[Mg^{2+}]_m$ increased more than twofold – ranging from 12 mM (control) to ~26 mM (Figure 9B), but with stable $\Delta[Mg^{2+}]$ (~9.8 mM, Figure 10B). At all conditions, muscle NPS remained around 14.4 µg/mg fresh weight (Figure 9B), with no differences on CTO (~24.5%).

For *M. acanthurus*, the RI was greater than 1 only for $\Delta[Na^+]$% (1.3, see Table 2). $[Total]_m$ was of ~248 mM at all conditions, with a steady ratio to hemolymph osmolality of ~0.48 (see Table 1). Only chloride concentrations had a significant correlation between ECF and ICF ($R^2$=0.52, $[Cl^-]_m = 0.16 \times [Cl^-]_h + 16$, Figure 11B).

### 3.4. *Aegla schmitti*

Osmolality of the hemolymph in *A. schmitti* increased from 430 mOsm/kg $H_2O$ on freshwater control group to ~696 mOsm/kg $H_2O$ at 25‰ salinity. The osmotic challenges did not influence tissue hydration, which remained stable close to 85% at all groups (Figure 2D).

$[Na^+]_h$ progressively increased, ranging from 209 mM (control) to 260 and 377 mM after 5 and 10 days on brackish water. $[Na^+]_m$ greatly increased at both hyper-osmotic challenges (57 mM on control freshwater animals to ~130 mM. Figure 7C), but

CTO did not alter significantly (~14.8%). $\Delta[Na^+]$ increased ~110 mM at the longest exposure period (Figure 8C).

Similarly, $[Cl^-]_h$ increased more than twofold on hyper-osmotic conditions, while $[Cl^-]_m$ increased almost three times, ranging from 62 mM (control) to ~178 mM (Figure 7D), with an increase of ~8% on its CTO. $\Delta[Cl^-]$ ranged from -104 mM at control and 5 days group to -171 mM at the longest exposure (Figure 8D).

At all groups, $[K^+]_h$ remained steady around 9.9 mM, and an increase on $[K^+]_m$ was observed (75 to ~110 mM,, Figure 9C), reflected on a marked increase of $\Delta[K^+]_h$ (~40 mM on the longest period, Figure 10C). The ion's CTO was steady around 14.4%.

$[Mg^{2+}]_h$ increased almost four times on the hyper-osmotic challenges, ranging from 6.1 mM (freshwater) to ~24.2 mM at both experimental groups. $[Mg^{2+}]_m$ ranged from 13.8 mM on control freshwater animals (7.7 mM higher than on the hemolymph), to ~19 mM on brackish water, being isoinic to $[Mg^{2+}]_h$ (Figures 9D and 10D). NPS on the tissue increased almost twofold, going from 9.4 to ~18 μg/mg fresh weight (Figure 9D), but always contributing to approximately 22% of tissue osmolality (see Table 1).

For sodium, chloride and potassium, RI shows that variations on the ICF were much greater than on the ECF (Table 2). $[Total]_m$ increasing more than twofold on the hyper-osmotic challenges, ranging from 202 to ~425 mM after 5 days at 25‰ salinity. The ratio of $[Total]_m$ to hemolymph osmolality was the greatest at the 5 days group (0.66). Sodium ($R^2$=0.29, $[Na^+]_m = 0.288 \times [Na^+]_h + 23.55$, Figure 11A), chloride ($R^2$=0.7, $[Cl^-]_m = 0.624 \times [Cl^-]_h - 26.6$, Figure 11B) and magnesium ($R^2$=0.31, $[Mg^{2+}]_m = 0.305 \times [Mg^{2+}]_h + 11.96$, Figure 11D) of muscle and hemolymph were positively correlated for this species.

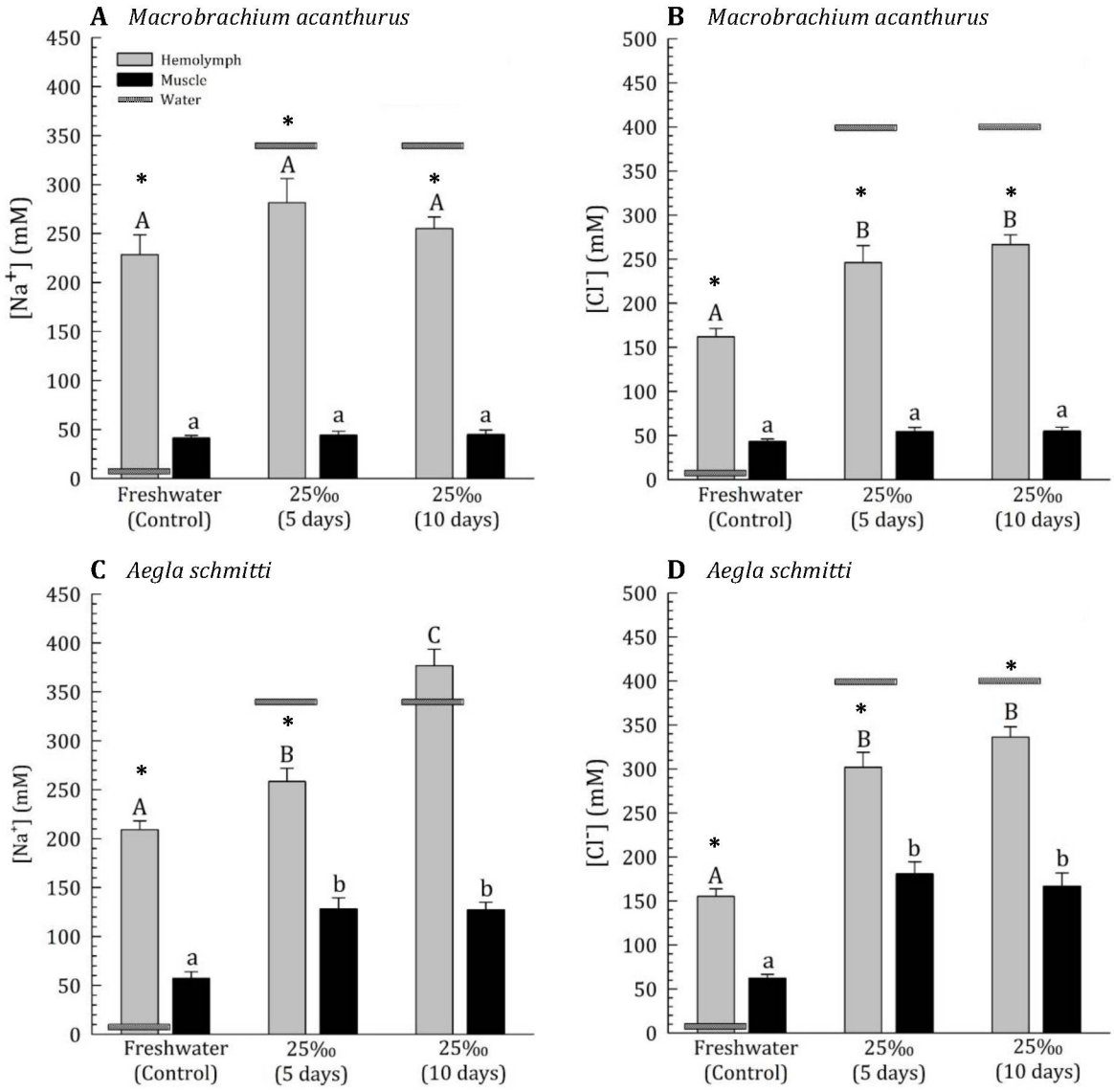


**Figure 7.** Profiling of sodium and chloride on hemolymph and muscle of *Macrobrachium acanthurus* and *Aegla schmitti* submitted to hyper-osmotic challenges. Sodium (**A, C**) and chloride (**B, D**) of *Macrobrachium acanthurus* (n=10) and *Aegla schmitti* (n=10), respectively. Different upper and lowercase letters stand for statistically different values for hemolymph and muscle concentrations. Horizontal marks are a reference to calculated concentration of the ion on the water, based on Prosser (1973), and asterisks indicate a significant difference between hemolymph and water concentrations, reflecting hyper- or hypo-regulation.

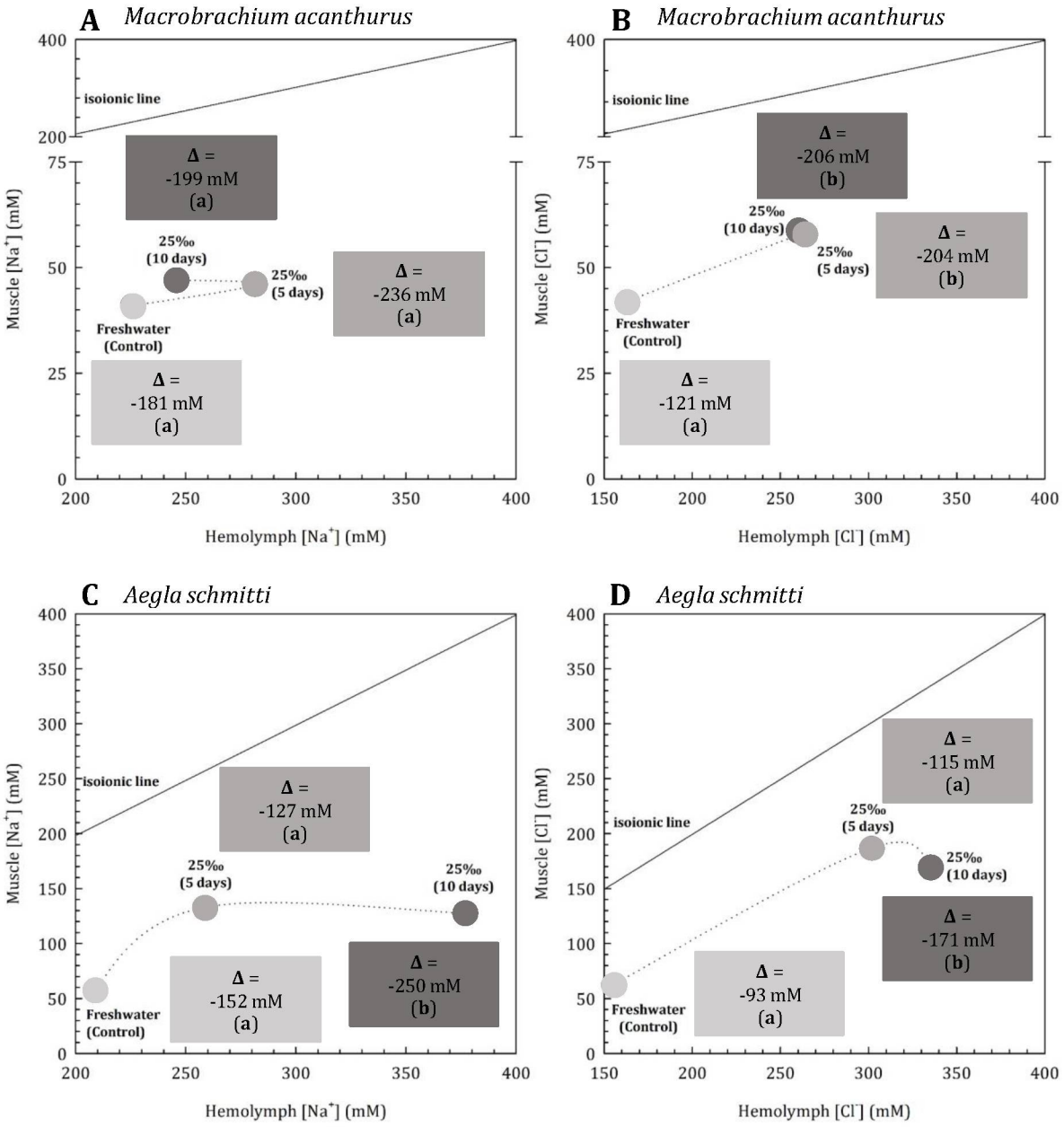


**Figure 8.** Differences of sodium and chloride between muscle and hemolymph of *Macrobrachium acanthurus* and *Aegla schmitti* submitted to hypo-osmotic challenges. Sodium (**A, C**) and chloride (**B, D**) of *Macrobrachium acanthurus* (n=10) and *Aegla schmitti* (n=10), respectively. Δ values are $[ion]_{muscle} - [ion]_{hemolymph}$. Different lowercase letters stand for statistically different values of Δ among groups. Only mean values are expressed.

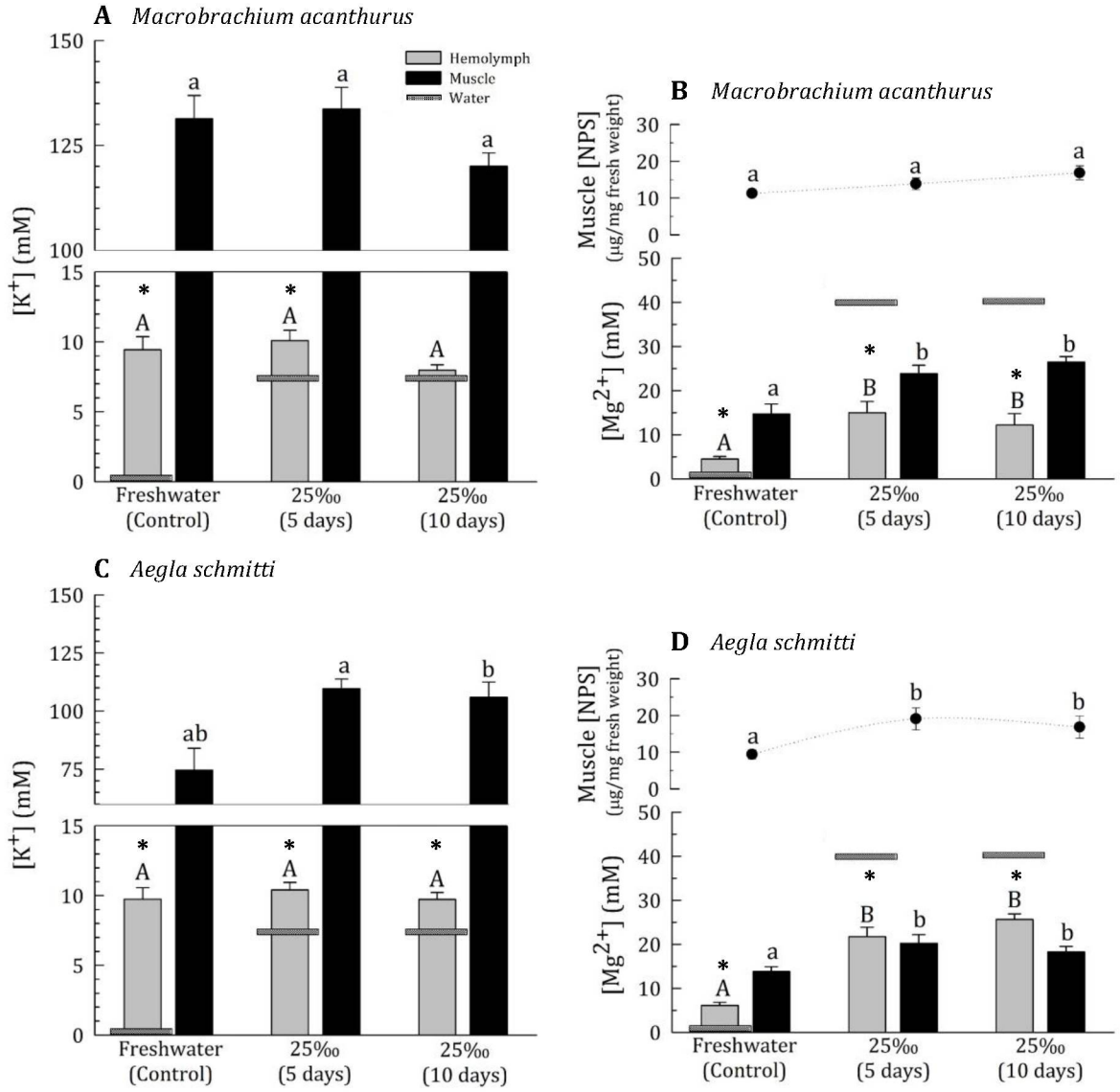


**Figure 9.** Profiling of potassium and magnesium on hemolymph and muscle (and muscle NPS) *Macrobrachium acanthurus* and *Aegla schmitti* submitted to hypo-osmotic challenges. Potassium (**A, C**) and magnesium and NPS (**B, D**) of *Macrobrachium acanthurus* (n=10) and *Aegla schmitti* (n=10), respectively. Different upper and lowercase letters stand for statistically different values for hemolymph and muscle concentrations. Horizontal marks are a reference to calculated concentration of the ion on the water, based on Prosser (1973), and asterisks indicate a significant difference between hemolymph and water concentrations, reflecting hyper- or hypo-regulation.

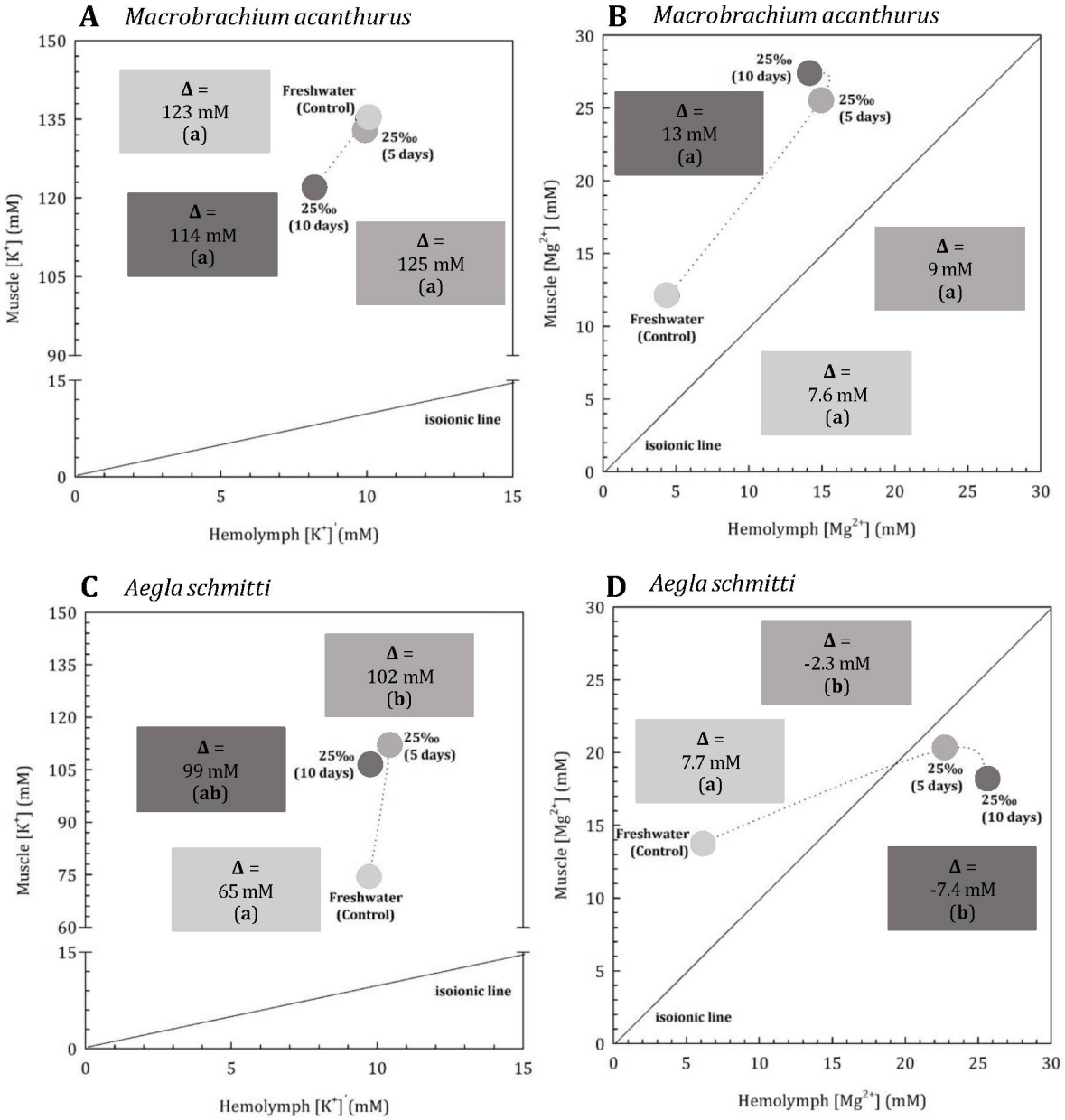


**Figure 10.** Differences of potassium and magnesium between muscle and hemolymph of *Macrobrachium acanthurus* and *Aegla schmitti* submitted to hypo-osmotic challenges. Potassium (**A, C**) and magnesium (**B, D**) of *Macrobrachium acanthurus* (n=10) and *Aegla schmitti* (n=10), respectively. Δ values are $[ion]_{muscle} - [ion]_{hemolymph}$. Different lowercase letters stand for statistically different values of Δ among groups. Only mean values are expressed.

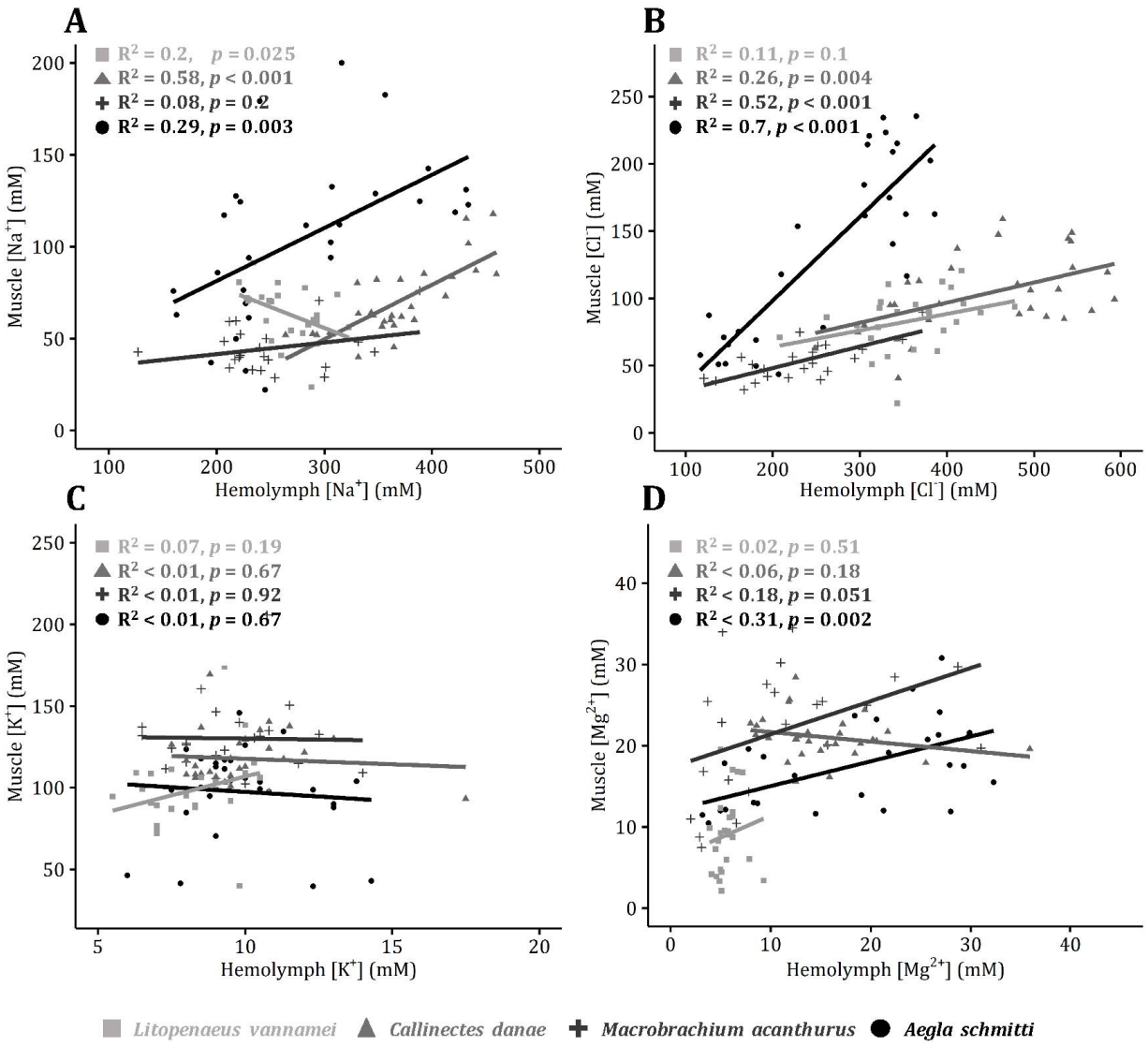


**Figure 11.** Correlations between hemolymph and muscle sodium (**A**), chloride (**B**), potassium (**C**) and magnesium (**D**), of *Litopenaeus vannamei* (■ ), *Callinectes danae* (▲), *Macrobrachium acanthurus* (▮), and *Aegla schmitti* (● ), pooling animals of both control and osmotic challenge groups. Equations for significant correlations ($p<0.05$) are expressed on the text.

**Table 1.** Contribution to tissue osmolality (CTO, %), sum of inorganic ions ($[Total]_m$) and its ratio to hemolymph osmolality ($[Total]_m/Osm_{hemolymph}$) on muscle of four decapod crustacean species. FW = freshwater. Lowercase letters (boxes highlighted in light orange) indicate significant differences ($p<0.05$) of the specific parameter among experimental conditions. Data presented are mean ± standard deviation.

| | Species | Experimental condition | Contribution to tissue osmolality (%) | | | | $[Total]_m$ (inorganic, mM) | $[Total]_m/Osm_h$ |
|---|---|---|---|---|---|---|---|---|
| | | | $[NPS]_m$ | $[Na^+]_m$ | $[Cl^-]_m$ | $[K^+]_m$ | | |
| Hypo-osmotic challenge | *Litopenaeus vannamei* | **25‰, control** | 33.1 ± 8.5$^a$ | 6.6 ± 1.9$^a$ | 8.8 ± 2.7$^a$ | 12.4 ± 4.2$^a$ | 254 ± 69$^a$ | 0.38 ± 0.11$^a$ |
| | | **15‰, 5 days** | 35.1 ± 9.6$^a$ | **8.3 ± 2$^{ab}$** | 11.6 ± 3.7$^a$ | 11.4 ± 1.5$^a$ | 240 ± 38$^a$ | 0.39 ± 0.07$^a$ |
| | | **15‰, 10 days** | 30.8 ± 9.1$^a$ | **10.3 ± 1.4$^b$** | 12.3 ± 2.4$^a$ | 13.6 ± 1.3$^a$ | 267 ± 27$^a$ | 0.45 ± 0.05$^a$ |
| | *Callinectes danae* | **30‰,control** | 16.5 ± 5.4$^a$ | 7.3 ± 1.9$^a$ | **10.4 ± 2.3$^{ab}$** | 10.6 ± 1.1$^a$ | 347 ± 42$^a$ | 0.38 ± 0.04$^a$ |
| | | **15‰, 5 days** | 21 ± 9.7$^a$ | 7 ± 1.4$^a$ | 11.3 ± 1.8$^a$ | 11.1 ± 1$^a$ | **310 ± 36$^b$** | 0.39 ± 0.04$^a$ |
| | | **15‰, 10 days** | 22.3 ± 3.5$^a$ | 6.1 ± 1.8$^a$ | **8.7 ± 2.3$^b$** | **13.74 ± 1.9$^b$** | **275 ± 33$^b$** | 0.39 ± 0.04$^a$ |
| Hyper-osmotic challenge | *Macrobrachium acanthurus* | **FW, control** | 24.8 ± 6.9$^a$ | 7.8 ± 2.4$^a$ | 8 ± 2.1$^a$ | 25.3 ± 7.1$^a$ | 228 ± 37$^a$ | 0.54 ± 0.12$^a$ |
| | | **25‰, 5 days** | 21.2 ± 8.3$^a$ | 5.9 ± 1.2$^a$ | 7.4 ± 1.1$^a$ | **17.6 ± 2.2$^b$** | 264 ± 45$^a$ | 0.45 ± 0.04$^a$ |
| | | **25‰, 10 days** | 27.8 ± 8.4$^a$ | 6.9 ± 2.1$^a$ | 8.5 ± 2.2$^a$ | **17.8 ± 1.4$^b$** | 254 ± 35$^a$ | 0.47 ± 0.06$^a$ |
| | *Aegla schmitti* | **FW, control** | 19 ± 5.8$^a$ | 12.6 ± 6.8$^a$ | 13.1 ± 5.2$^a$ | 16 ± 8.6$^a$ | 202 ± 48$^a$ | 0.51 ± 0.2$^a$ |
| | | **25‰, 5 days** | 25.3 ± 14$^a$ | 17 ± 4.2$^a$ | **23.5 ± 3.4$^b$** | 14.3 ± 2.3$^a$ | **432 ± 89$^b$** | **0.66 ± 0.07$^b$** |
| | | **25‰, 10 days** | 21.9 ± 13.1$^a$ | 15.1 ± 3.3$^a$ | **20 ± 6.6$^b$** | 12.9 ± 2.6$^a$ | **423 ± 55$^b$** | **0.59 ± 0.08$^{ab}$** |

**Table 2.** Ratio between relative variation (Δ%) of assessed inorganic ions on hemolymph and muscle of the four decapod species submitted to osmotic challenges. Δ% was calculated in function of respective control group for each species. Hemolymph Δ% represents a numerical approach to the crustacean's ability to perform Anisosmotic Extracellular Regulation (AER), and muscle Δ%, IIR, considering the salinity challenges posed. The 'Regulatory Index' shows how ionic variations on hemolymph and muscle differed from each other. (the greater than 1, greater might be the influence of IIR on measured hemolymph concentrations). Values highlighted in orange are those greater than one. Missing values (-) are divisions by zero.

| Species | Experimental condition | Hemolymph Δ% ('AER') | | | | Muscle Δ% ('IIR') | | | | Regulatory Index (IIR/AER) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $Na^+$ | $Cl^-$ | $K^+$ | $Mg^{2+}$ | $Na^+$ | $Cl^-$ | $K^+$ | $Mg^{2+}$ | $Na^+$ | $Cl^-$ | $K^+$ | $Mg^{2+}$ |
| *Litopenaeus vannamei* | **15‰, 5 days** | -12 | -2 | -24 | 0 | 9 | 13 | -21 | -45 | 0.7 | **7.6** | 0.9 | - |
| | **15‰, 10 days** | -21 | 9 | -27 | 2 | 30 | 14 | -9 | -37 | **1.4** | **1.6** | 0.3 | **20.5** |
| *Callinectes danae* | **15‰, 5 days** | -14 | 3 | 2 | -30 | -18 | -7 | -11 | -1 | **1.2** | **2.5** | **5.4** | 0.0 |
| | **15‰, 10 days** | -23 | -33 | -9 | -43 | -36 | -36 | 0 | 8 | **1.5** | **1.1** | 0.0 | -0.2 |
| *Macrobrachium acanthurus* | **25‰, 5 days** | 27 | 61 | 2 | 218 | 12 | 38 | 2 | 112 | 0.5 | 0.6 | 0.7 | 0.5 |
| | **25‰, 10 days** | 11 | 62 | -17 | 222 | 15 | 38 | -8 | 126 | **1.4** | 0.6 | 0.5 | 0.6 |
| *Aegla schmitti* | **25‰, 5 days** | 24 | 95 | 8 | 272 | 133 | 202 | 49 | 48 | **5.5** | **2.1** | **6.0** | 0.2 |
| | **25‰, 10 days** | 80 | 119 | 0 | 323 | 123 | 173 | 45 | 33 | **1.5** | **1.4** | - | 0.1 |

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# 4. DISCUSSION

## 4.1. *Litopenaeus vannamei*

The white leg shrimp *L. vannamei* is a very euryhaline species of the Penaeidae family, in which adult individuals knowingly inhabit coastal and marine environments, being able to tolerate wide salinity ranges. They are very euryhaline, and display a strategy of efficient hyper-hypo osmoregulation, that is, preventing intense changes in hemolymph osmolality when submitted to salinity changes. In fact, different attempts on rearing of this species on inland waters of low salinities have succeeded (Chong-robles et al., 2013; Gomez-Jimenez et al., 2004; Madrigal et al., 2018), and the white leg shrimp is the most commonly cultivated species on the world (Zhou et al., 2011). The ability to maintain relatively constant osmolality of the hemolymph could be observed on the present work: when challenged with salinity reduction from 25 to 15‰ salinity (~300 mOsm/kg $H_2O$ challenge), its hemolymph osmolality decreased by ~80 mOsm/kg $H_2O$, after 5-10 days, thus already under steady-state conditions.

Interestingly, hemolymph dilution was observed for $[Na^+]_h$, while $[Cl^-]_h$ remained unchanged. If we were to consider this steadiness of $[Cl^-]_h$ as a consequence of its efflux from intracellular compartments due to RVD, it would likely be accompanied by the detection of decreased $[Cl^-]_m$ on muscle, which was not the case. In fact, even a slight increase was detected after 5 days in 15‰ salinity, but the ion's CTO was stable throughout all experiments. This is reflected on the ion's gradient between ICF and ECF, which also did not change, in contrast to what was observed for sodium (see figure 4A and 4B). Yet, only $[K^+]_m$ had a relative decrease on hypo-osmotic conditions, as it would be expected: potassium channels play an important role on RVD, leading to its efflux alongside with intracellular water (Amado et al., 2006; Foster et al., 2010, Kirschner, 1991). This effect has already been studied several times with isolated muscle tissue and using channel blockers such as barium chloride (Freire et al., 2008a; Ruiz and Souza, 2008; Amado et al., 2015; Castellano et al., 2016).

More interestingly, one would expect that the main decrease on tissue osmolytes would happen with NPS, but this was not detected in this study for *L. vannamei*. Free amino acids are the main intracellular osmolytes, especially in marine decapod species (Wheatly, 1985; Faria et al., 2011). Its synthesis (or in this particular case,

efflux/catabolism) is a long-term response to osmotic stress (Charmantier et al., 2009; Foster et al., 2010), while the movement of inorganic osmolytes is considered an immediate response to adjust cellular volume to the 'new' osmolality of the hemolymph. This behaviour could perhaps be elicited if the chosen experimental times were shorter: in Foster et al. (2010), muscle NPS of four decapod species showed significant changes throughout a course of 24 hours, as a response to osmotic challenges.

Since the animals in this study were challenged for 5 and 10 days, this may be the reason for the lack of detection on change in muscle NPS. Also, *L. vannamei* was the only species in this work that was not able to maintain tissue hydration during the osmotic challenge. MWC, as measured, can be interpreted as a proxy to cell volume regulation, and how well an animal is able to perform it. Efficient hyper-hypo-regulators such as *L. vannamei*, which are able to keep ECF osmolality at narrow ranges, might be weak volume regulators when compared to euryhaline osmoconformers. This trend has been suggested for decapod crustaceans (Freire et al., 2008a; Foster et al., 2010) on isolated muscle tissues. In these cited studies, the marine osmoconformer species studied (the boxcrab *Hepatus pudibundus*) had a better performance at maintaining tissue hydration than the strong freshwater hyper-regulators *Macrobrachium acanthurus* and *Dilocarcinus pagei*.

In this sense, an euryhaline osmoconformer would require fine tuning of IIR mechanisms to avoid cells from being damaged (Kirschner, 1991; Freire et al., 2008b; Charmantier et al., 2009; Foster et al., 2010), since ECF osmolality is not buffered by osmoregulatory processes when facing fluctuations of environmental salinity. Since the opposite was observed to *L. vannamei*, it is safe to assume that even though the animal is able to stall ECF dilution on hypo-osmotic conditions – by hyper-regulating – the species has a lower capacity to perform IIR.

To 'adequately' perform IIR and guarantee steady tissue hydration on the new state of lower hemolymph osmolality, we would expect that muscle ionic concentrations would reduce accordingly, maintaining the ratio between ECF and ICF compartments. Indeed, $[Total]_m/Osm_h$ did not alter on hypo-osmotic conditions, but interestingly neither $[Total]_m$. Perhaps these variations could not be detected by the statistical analysis perfomed, since a higher variability can be observed for control and 5 days groups, in

relation to the longest hypo-osmotic challenge – which shows an apparent higher average and tighter standard deviation for these parameters (see Table 1).

More specifically, lower $[Cl^-]_m$ and $[K^+]_m$, as mentioned before, were expected to be detected, given the role of chloride and potassium channels on RVD. Indeed, this was valid only for $[K^+]_m$. This seemingly 'insufficient' ionic efflux, to perform RVD might be reflected on the higher tissue hydration for the species. To support this idea, the relative IIR/AER index suggest that only $[K^+]_m$ decreased in accordance to the hemolymph, since both muscle Δ% and hemolymph Δ% are negative values, in contrast to sodium and chloride, which showed positive muscle Δ% – although not reflected in a significant increase on muscle concentrations (see Figures 3A and B). In this sense, the apparently mild efflux of $K^+$ from ICF had a minor role on both i) influencing detected $[K^+]_h$, since RI < 1; and ii) efficiently performing RVD, given the increase in MWC. These additional approaches to the data reinforce that *L. vannamei* did not express the necessary mechanisms to perform RVD, which might be in accordance to the putative lower capacity of IIR of efficient hyper-hypo-regulators (Foster et al., 2010).

### 4.2. *Callinectes danae*

The swimming crab *C. danae* occurs in marine and estuarine environments, and is considered – alongside with other species of *Callinectes* – a weak regulator, since it is able to hyper-regulate on brackish waters but cannot cope with freshwater (Péqueux, 1995; Freire et al., 2008b, 2011). When in full strength seawater, *C. danae* is essentially isosmotic to the environment, which is consistent to what was observed on control group crabs maintained at 30‰ (~903 mOsm/kg $H_2O$). When facing a dilution of 15‰ of environmental salinity, the swimming crab was able to maintain a gradient of ~300 mOsm/kg $H_2O$ with external medium, and its hemolymph osmolality decreased by 120-200 mOsm/kg $H_2O$ on these conditions. This suggests that the species might tolerate – and cope with – broad ranges of ECF osmolality, in accordance to the highly variable nature of estuarine environments where this species is commonly found (Mantelatto and Fransozo, 2000; Freire et al., 2011). Other species of *Callinectes* show different ranges of tolerance to seawater dilution: *C. ornatus* occurs in polyhaline portions of the estuary,

while *C. sapidus* can be found on oligohaline and even freshwater environments (Gerard and Gilles, 1972; Ettinger and Blye Jr, 1981; Freire et al., 2011).

Comparing with *L. vannamei*, *C. danae* had a relatively weaker performance on avoiding hemolymph dilution at lower salinities, but showed remarkable hyper-regulatory capability. In fact, up to 5 days on 15‰ salinity, the ionic profile of this species was similar to that of *L. vannamei*, in which decreased osmolality happened mainly because of reduced $[Na^+]_h$, while $[Cl^-]_h$ (~500 mM) remained unchanged. *C. danae* is able to survive at 10‰ salinity for more than 24h and a mortality of 50% after 6 hours on freshwater is observed (D. C. Bozza and L. P. Rios, personal communication). The long time of exposure to the hypo-osmotic challenge lead to such marked decreases on nominal ECF concentrations.

In addition to its efficiency on dealing with low salinities, *C. danae* also maintained muscle hydration stable at all conditions. Once again, the expected decrease of muscle NPS to perform RVD was not detected in this experiment, but both $[Cl^-]_m$ and $[Na^+]_m$ decreased ~35% of its control levels (with a slight decrease of their CTO, maintaining a positive correlation to ECF levels). As aforementioned, when facing hypo-osmotic conditions, cells tend to recruit potassium and chloride channels to promote their efflux and therefore reduce its intracellular concentration, leading to the loss of water, in ongoing RVD. This is consistent with the lower levels of $[Cl^-]_m$ here detected. However, $[K^+]_m$ was similar under all treatments. In 'its place', $[Na^+]_m$ was extruded from the tissue, which could suggest that alongside the typical potassium and chloride channels, long exposures to hypo-osmotic challenges could induce higher $Na^+/K^+$-ATPase (NKA) activity (Horisberger, 1991; Jorgensen et al., 2003; Amado et al., 2015), therefore recycling $[K^+]_m$. and maintaining its steady values. A similar trend could be observed for *L. vannamei*, where an initial drop on $[K^+]_m$ was detected.

Overall, the total inorganic concentration of *C. danae* muscle tissue lowered 40 to 70mM from control value at hypo-osmotic conditions, and MWC was maintained. This lower concentration indicates that the muscle tissue indeed secreted ions, especially $Cl^-$, which is supported by its reduced CTO (see Table 1). Since CTO is expressed as a percentage of the hemolymph osmolality – reflecting isosmoticity between ICF and ECF–, chloride CTO values lower than the observed for the control group suggest that

$[Cl^-]_m$ was the main ion secreted from the tissue – preferentially, perhaps – shifting the initial ionic balance between the two compartments.

In this sense, the efflux of NaCl from the muscle of *C. danae* is also reflected on the positive correlations drawn for these ions (see Figure 11) and the relative Δ% of muscle and hemolymph concentrations, given that both are negative, indicating lower concentrations for ICF and ECF. Interestingly, the greatest RI observed for these ions was for chloride, on the shorter exposure to dilute seawater: in that case, the observed $[Cl^-]_h$ was similar to the control, seawater group (see Figure 3D), while hemolymph osmolality already decreased by ~120 mOsm/kg $H_2O$, mainly due to lower $[Na^+]_h$. In this sense, the dissociation of $Na^+$ and $Cl^-$ on the specific 5 days exposure could also be related to $[Cl^-]_m$ efflux of RVD.

The capacity of maintaining tissue hydration (and thus cell integrity) at such wide ranges of ECF osmolality is an important aspect of this species euryhalinity. We may say that the result of *C. danae* (less efficient osmoregulator when compared to *L. vannamei*) and *L. vannamei* is compatible with the hypothesis that more regulating decapods lose some capacity to regulate tissue water/volume when salinity challenged (Foster et al., 2010). In this sense, the weak hyper-regulator/conformer *C. danae*, which dwells both on marine and diluted estuarine waters, 'still' relies on IIR mechanisms to properly deal with intense salinity fluctuations, since it's AER capability, as demonstrated here, tends to weaken over time.

### 4.3. *Macrobrachium acanthurus*

The freshwater prawn *M. acanthurus* is a strong hyper-regulator of diadromous behaviour, requiring brackish waters for early larval development. "Strong osmoregulation" is meant in the sense of Kirschner (2004), and as cited in Freire et al. (2008a,b), Charmantier et al. (2009), Henry et al. (2012). It refers to freshwater crustaceans that are able to sustain large osmotic gradients (300-600 mOsm/kg $H_2O$), displaying very low cutaneous and branchial osmotic and ionic permeabilities, absorbing NaCl from freshwater through their gills or other epithelia with the powering of the V-$H^+$-ATPase, and eventually producing dilute urine, also obtaining salt from their food. The Palaemonidae family is considered of recent freshwater invasion, thus many (of

especially coastal riverine distribution) representatives of this family and of *Macrobrachium* are diadromous, such as *M. acanthurus* and *M. olfersi*, which occur along the South American coast (Anger, 2016). Other congeneric species such as *M. potiuna* are completely hololimnetic (Albertoni et al., 2002; Freire et al., 2003, 2018; Collins et al., 2011; Anger, 2016). This trait of the evolutionary history of *M. acanthurus* is reflected in its strong euryhalinity, which has been extensively studied over the last decades.

Here, *M. acanthurus* displayed an increase in hemolymph osmolality of only ~100 mOsm/kg $H_2O$ while facing brackish waters of 25‰ (a shift from ~15 to 750 mOsm/kg $H_2O$ in external osmolality), confirming its tendency of hypo-conformation or hypo-regulation under hyper-osmotic conditions (Maraschi et al., 2015; Freire et al., 2017).

To deal with such steep osmotic gradients, possibly a response of reducing or shutting down salt absorption on gills could take place. However, in Freire et al. (2018), individuals of this species submitted to 20‰ for up to five days showed no reduction of neither NKA nor carbonic anhydrase activities from their gills. Interestingly, this seems to be an early response, possibly during the initial hours of exposure to the hyper-osmotic shock, given that Maraschi et al. (2015) also found no differences on gill expression of NKA after 24 hours on 25‰ salinity. However, NKA activity is also related to hypo-regulating mechanisms on gill, acting along with the co-transporter NKCC (Freire et al., 2008b; Henry et al., 2012; McNamara and Faria, 2012), which could hinder our interpretation of simple reduction of salt uptake via lower NKA activity. Here, the hemolymph osmolality of *M. acanthurus* rose ~36%, similar to what was observed in Freire et al. (2018), which, in this sense, could suggest that not shutting down salt absorption, but rather reducing it, probably was an elicited response by the prawns in this experiment.

*M. acanthurus* displayed tight hyper-hypo-regulation of $[Mg^{2+}]_h$: despite a threefold increase, from 4 to 14 mM. At the hyper-osmotic challenge, values were still below water levels of, which are expected to be of ~40 mM. The maintenance of $[Mg^{2+}]_h$ at such narrow ranges and low concentrations is a trend observed for all species in this study, where hemolymph concentrations were far from being iso-ionic to the water, as a result of putative antennal gland magnesium regulation (Charmantier et al., 2009; Freire et al., 2003, 2008b).

Given what was observed and previously reported in Foster et al. (2010), Freire et al. (2018) and Maraschi et al. (2015), a decrease on MWC was expected to happen for this species on such strong salinity challenge. Curiously, despite an obvious tendency of decreased tissue hydration after 5 days (from 79 on freshwater, to 76%, then back to 79% after 10 days), no significant differences were supported by the statistical analysis performed. However, differently from what was observed for *C. danae*, $[Total]_m$ did not vary, being stable around ~248 mM. This trend was reflected on the analysis of individual osmolytes, since their net concentrations and CTO were similar at all conditions (see Table 1 and Figures 7A and 7B).

On the opposite direction of what was expected for the marine species *L. vannamei* and *C. danae*, to keep MWC stable on hyper-osmotic conditions, we would expect an increase on muscle concentrations, due to ionic influx of RVI. However, similarly to what we observed for *L. vannamei*, *M. acanthurus* was able to maintain a tight range of hemolymph osmolality in the proposed salinity challenges, allowing, as mentioned before, an increase of only ~100 mOsm/kg $H_2O$. In this sense, the tissues of *M. acanthurus* possibly were not challenged to the point of triggering a strong response of RVI, in which we could possibly detect a marked increase on individual ions, NPS or $[Total]_m$. This could be a good observation of how AER can buffer ECF osmolality under salinity challenges, thus protecting the tissues from greater osmolality fluctuations and diminishing the demand for IIR (Kirschner, 1991; Charmantier et al., 2009).

In addition, a study conducted in Freire et al. (2013) on isolated muscle of *M. acanthurus* (and several other species) exposed to hyper-osmotic saline (600 mOsm/kg $H_2O$) revealed that the specific inhibitor of the NKCC cotransporter, furosemide, did not affect tissue hydration. NKCC is a major effector on RVI, promoting influx of $Na^+$, $K^+$ and 2 $Cl^-$ to the ICF. This suggests that the expression of the NKCC on muscle of *M. acanthurus* is minimal or on-existent, therefore its role on RVI could be negligible in this situation. Other transporters, such the $Na+/H^+$ and $Cl^-/HCO_3^-$ exchangers might also act during RVI (Amado et al., 2006), which could perhaps be the case for this species. On the other hand, if *M. acanthurus* were in fact actively performing RVI during these challenges, it could be in accordance to the observed lack of higher $[K^+]_m$, but the IIR/AER < 1 for this ion suggests that this was not the case.

In relation to variations on hemolymph and muscle concentrations, while the hyper-osmotic challenge lead to a relative increase of 27 and 11% on $[Na^+]_h$ (5 and 10 days, respectively) and ~61% for $[Cl^-]_h$ muscle levels for theses ions increased ~13 and 38% (Table 1), roughly half of what was observed for the hemolymph, which is reflected on the RI, generally lower than 1 (see Table 2). At the longest exposure to brackish water, RI for $Na^+$ is 1.35, in contrast to the other assessed ions on both 5 and 10 days, indicating that possibly, at this specific situation, the sodium influx on RVI could have influenced $[Na^+]_h$ (which curiously, had the highest average among experimental groups). If, in addition to this, we had detected significantly higher $[Na^+]_m$, a putative role of the $Na^+/H^+$ exchanger on muscle RVI could be proposed, which could possibly be detected under stronger salinity challenges than the ones posed in this study. Other than that, our results suggest that at the given salinity, the role of RVI on reducing hemolymph concentrations on *M. acanthurus* was minimal, possibly restricted to the adjustment of tissue hydration through the influx of sodium.

### 4.4. *Aegla schmitti*

The anomuran *A. schmitti* is completely hololimnetic, restricted to continental freshwaters. Despite having a longer evolutionary history on freshwater than the palaeomonid *M. acanthurus*, *A. schmitti* could also remarkably cope with such long and strong hyper-osmotic challenges. In comparison to other aeglids such as *A. franca* and *A. longirostri*, *A. schmitti* is the most salt tolerant (Faria et al., 2011; Bozza et al., 2019). As *M. acanthurus*, this aeglid hyper-regulates in freshwater, maintaining a strong gradient of ~400 mOsm/kg $H_2O$, such as reported in this work. After the hyper-osmotic challenge, the animals seemed to hypo-regulate up to five days (hemolymph 74 mOsm/kg $H_2O$ lower than the water), but entered isosmoticity at the longest exposure time (716 mOsm/kg $H_2O$, considering a 95% confidence interval on the isosmotic point of 750 mOsm/kg $H_2O$ at 25‰ salinity). Interestingly, this 'hypo-iso' pattern was mirrored only by $[Na^+]_h$, while $[Cl^-]_h$ seemed to be hypo-regulated after 10 days at 25‰ (340 mM, in contrast to calculated ~400 mM on the water). As argued in Bozza et al. (2019) – in which the 'hyper-hypo' pattern was reported for both sodium and chloride -, the apparent hypo-regulation of these ions might be strongly related to RVI. Similar to what was discussed for *M. acanthurus*, any expected decrease in gill NKA activity could take place, but Bozza

et al. (2019) did not find any difference on its activity when aeglids were exposed to same salinities as in this work, and possible shutdown on high-affinity salt uptake systems would not take place after such long periods on hyper-osmoticity. Again, this might also be in accordance with the enzyme's role on hypo-regulation.

$[K^+]_h$, on the other hand, was always tightly hyper-regulated, with steady values at ~10mM. The antennal glands, especially on freshwater crustaceans, are responsible for its reabsorption and thus maintenance of gradient to $[K^+]_m$ for proper IIR function (Freire et al., 2008b). When taken in consideration, the results for $[K^+]_h$ account for the proposed osmoconformity at higher salinities, yet maintaining regular composition of ECF. $[Mg^{2+}]_h$ was both hyper - (freshwater) and hypo-regulated, and similar results have been reported for other freshwater decapods exposed to high salinities(Wheatly and Henry, 1987; Freire et al., 2003; Faria et al., 2011).

*A. schmitti* was able to sustain muscle hydration ~84% at all conditions, and apparently, it was achieved by a strong rise on total tissue concentration, that increased twofold from 202 mM to ~423 mM on higher salinity. All inorganic ions and NPS were more concentrated at the hyper-osmotic conditions. For *Aegla franca*, de Faria et al. (2011) reported a threefold increase of muscle free amino acids when the animals were exposed to 25‰ salinity, keeping steady concentrations for up to 10 days. Regardless of the differences between the NPS methodology and indivual analysis of the amino acid pool on muscle tissue, here we evidenced that for *A. schmitti* on the same hyper-osmotic condition, muscle NPS increased twofold. Seemingly, this could be a consequence of the nutritional status of the animals on each experiment, since in Faria et al. (2011) animals were fed during the experiments, while here they were not. In this plausible scenario, the rates of oxidation and synthesis of amino acids and protein metabolism, other than its sheer transport across cell membrane, could be altered (Foster et al., 2010; Gerard and Gilles, 1972; Gilles and Péqueux, 1981).

Curiously, $[Cl^-]_m$ had the most marked increase on both net concentration and CTO, in addition to the strongest reported correlation to $[Cl^-]_h$. A study conducted in Freire et al. (2013), with isolated muscle slices of *A. parana* exposed to furosemide (NKCC blocker), points out that this co-transporter plays an important role on muscle RVI, which could be extrapolated for *A. schmitti*. Considering the stoichiometry of NKCC ($1Na^+ : 1K^+ : 2Cl^-$), our results suggests that RVI on *A. schmitti* might be achieved not only

by the typical ion influx of NKCC, despite its clear relevance on volume regulation (Kirschner, 1991; Russell, 2000; Charmantier et al., 2009). The ratio of sodium, chloride and potassium increases (when compared to freshwater control values), at 25‰ salinity, are ~$2Na^+$ : $1K^+$ : $3Cl^-$, which interestingly is also in accordance to the NKCC stoichiometry on different cell types (Russell, 2000). This calculated ratio, however, is biased by the method used to evaluate the ionic content of muscle tissue, and did not have the aim to address this specific topic, so no conclusions on specific stoichiometry should be drawn from our results. However, NKCC indeed could play a major role on RVI for *A. schmitti*.

For the three other species of this work, the ratio between $[Total]_m/Osm_h$ was maintained throughout all experimental conditions (see Table 1). This might be a signal that the osmotic challenges posed to those animals did not reflect a challenging condition to their tissues, therefore not actively requiring strong, marked transport of ions to and from the ICF. Only *A. schmitti* presented a shift on this ratio, pointing out that an efficient, accumulation of osmolytes on muscle tissue happened, to counteract increased hemolymph osmolality. The salinity chosen for this experiment is close to the limits of salt tolerance for aeglids: Bozza et al. (2019) report 89% survival of *A. schmitti* after one day in seawater, and ~90% survival at 25‰ for up to 10 days. *A. franca* has a survivability of only 25% at 28‰, also for 10 days (Faria et al., 2011), and *A. longirostri* is even more sensitive to environmental salt, with no survivors after a 4 days exposure to 25‰ salinity (Cogo and Santos, 2007). This salinity 'threshold' (~25‰) for survival of these aeglids might be suited to elicit such extreme responses as the marked increases on tissue osmolytes.

In summary, *A. schmitti* apparently struggled, when compared to *M. acanthurus*, to cope with the proposed hyper-osmotic challenge. While concentrations on the hemolymph rose 24 and 80% for sodium and ~106% for chloride on higher salinity, this was accompanied by an average increase of ~127 and ~185% of $[Na^+]_m$ and $[Cl^-]_m$, respectively. Because muscle levels rose much greatly than what was observed for hemolymph (as reflected by remarkably high IIR/AER ratios, Table 2), and considering that the contribution of $[Cl^-]_m$ was greater at the hyper-osmotic challenge, apparently, RVI influence on reduced hemolymph concentrations are much more evident in this species than for the other decapods of this study.

## 5. CONCLUSIONS

We have here tested, using four distinct decapods, widely different in evolutionary history, habitat, habit, and osmoregulatory strategies, whether we could demonstrate that perhaps hemolymph ionic changes upon salinity challenges would not entirely be ascribable to anisosmotic extracellular regulation mechanisms. We stress that volume regulation mechanisms are always in place, and influences any observed ion concentration on hemolymph. It is not proposed to be a matter of 'kind' – if it happens or not –, but of 'degree', or how strongly IIR is capable of shifting the hemolymph ionic profile, under steady-state conditions, after 5-10 days of adjustment to a significant osmotic challenge.

Additional studies, using more direct methods of ion radioisotopes, intracellular electrodes, and transport protein imaging through immunocytochemistry could certainly provide a more conclusive answer to our initial question, and even provide a more quantitative picture of *in vivo* ionic fluxes between the internal compartments during osmoregulatory challenges. Despite the indirect and simple methods here employed, results pointed to the need of, in fact, considering fluxes of ions between EC and IC compartments when evaluating ECF changes and osmoregulatory capacities in crustaceans, in general, and decapods in particular.

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